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IV

No 1

[From THE JOURNAL OF HYGIENE, Vol. V. No. 1, January, 1905.]

FURTHER OBSERVATIONS ON A LEPROSY-LIKE DISEASE OF THE RAT.

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The object of this paper is to record briefly the result of certain observations and experiments on a disease of the rat, which is chiefly interesting from the close resemblance it bears to leprosy in the human subject. Not only are the lesions similar in their macroscopical and microscopical appearances, but the causal bacillus closely resembles the *Bacillus leprae* in its morphological characters, distribution in the tissues, staining reactions, and refusal to adopt a saprophytic habit of life.

A large number of acid-fast organisms have been isolated and studied within the past few years; from smegma, dung, various grasses, milk, butter, and nasal mucus, etc. However, only a few organisms belonging to this group have been observed which bear a causal relation to naturally occurring diseases among animals. Indeed these may be limited to *B. tuberculosis*, the bacillus of Verruga of Peru, *B. leprae*, and certain acid-fast organisms of the streptothrix group giving rise to somewhat ill-defined diseases.

Until this condition in the rat was observed no disease was known in any of the lower animals which pathologically or bacteriologically had any very close resemblance to leprosy in man.

The disease was first observed by Stefansky (1903), while working at plague in Odessa. He found that 5 % of the rats (*Mus decumanus*) destroyed suffered from a condition affecting the skin and underlying muscles, and the lymphatic glands. Associated with the lesions, and apparently the causal agent, was a bacillus belonging to the acid-fast group. Stefansky gave an excellent description of the chief features of the disease. Rabinowitsch (1903), who had been working at Odessa during the time that Stefansky made his observations, on returning to Berlin was able to confirm Stefansky's results, for the rats there.

The disease had been observed in England and its nature recognised by the writer (1903) before the appearance of Stefansky's paper, and the experiments commenced in February, 1903, have been continued up to the present time. Since the first case was noted here, six other well marked examples of the disease, as it occurs naturally in the rat, have come under observation. Stefansky, Rabinowitsch, and the writer all stated that the animals which had been inoculated up to the time of their reports showed no signs of having acquired the disease, but since that time the writer has succeeded in obtaining a number of positive inoculation results in the case of rats.

The Disease as it occurs naturally.

Stefansky pointed out that there were two chief types of the disease, the one affecting primarily the skin and musculature, and the other confined to the lymphatic glands. There is, however, no very strict line of demarcation between the two types.

Skin and Muscle Lesions.

Patches of alopecia are the most marked feature. These may be extensive, affecting in some cases almost the whole thorax and abdomen. Above the surface of these bare patches of skin, elevated bosses, or even nodules, the size of a bean are frequently present. The surface of these elevations may be ulcerated, usually at the most prominent point. Ulceration is not, however, an essential feature and appears to result in part at least from mechanical causes (Fig. 1, Plate VI).

On incising an area affected in this manner the skin appears greatly thickened (up to half an inch), due to the presence of a pale yellow infiltration which cuts with a clean, dry, cheese-like surface. In most cases there is little tendency to softening and breaking down of the material. A scraping from its surface, stained by Ziehl-Neelsen's method, shows enormous masses of bacilli, many of them lying within the cells. Microscopical examination of a section through the skin and subjacent tissues reveals appearances which are almost identical with those found in the skin in lepra. The epithelial layers of the skin are but little affected; a few bacilli may be found to have forced their way between, or even into, the epithelial cells. The subepithelial infiltration is found to consist of cells lying in a stroma of connective tissue. The cells in part consist of leucocytes of different forms including lymphocytes, and in part of connective tissue cells; the endothelial cells

taking a prominent part in the formation of this granulation tissue. These cells are round, oval, or polygonal with a large nucleus and abundant protoplasm.

In a section appropriately stained one is struck by the presence of an enormous number of acid-fast bacilli with which nearly the whole of the cells of the tissue are packed (Fig. 5, Plate VII). The same difficulty in deciding whether the groups of bacilli lie in the lymphatic spaces is found as in the case of leprosy, but some of the appearances observed can be most satisfactorily interpreted in this way.

There is no doubt, however, that the majority of the bacilli actually lie within cells, and where these cells are of the large endothelial type an appearance strikingly resembling lepra cells results. The sebaceous glands and hair roots have mostly disappeared; those remaining appear to be undergoing a process of atrophy.

In the subcutaneous connective tissue the most prominent feature is the disappearance of fat. The whole region is infiltrated with cells, for the most part filled with bacilli. Here and there are small rounded or oval areas in which all appearance of cellular structure has disappeared; only *débris*, fragments of cell nuclei, and enormous masses of bacilli being visible. In one case in which the mammary gland was well developed the connective tissue framework and lymphatics were extensively invaded by the bacilli, but none of these could be found within the acini or ducts.

An interesting feature of the disease is the manner in which the striated muscle is invaded by the bacilli. Between the muscle fibres are frequently seen groups of cells of the types already mentioned. As these foci increase in size they cause atrophy of the muscle fibres and at the same time some of the cells burst and the bacilli penetrate the muscle fibre. In the early stage the bacilli are seen lying in the fibre, later this becomes degenerated, loses its striation, and as the invasion progresses only its outline is indicated by a tightly packed mass of bacilli round the nucleus. Even the outline may be lost by fragments of muscle fibre becoming more or less spindle-shaped and thus presenting the appearance of large cells.

Lymphatic Gland Lesions.

The lesions next in importance to those of the skin occur in the lymphatic glands. The glandular enlargement may be widespread; the axillary, cervical, and inguinal glands may all be affected, or only one of these groups. The enlargement may be considerable (Fig. 2,

Plate VI). In one case, in which most of the glands were involved, some of these had reached the size of a small hazel-nut. On section of the glands there are neither nodules nor necrotic areas visible to the naked eye.

Microscopical examination. The capsule may be thickened and may contain great masses of bacilli. The chief invasion of the gland itself appears to be along the sinuses, but where the disease is advanced the whole gland may be invaded. Along the sinuses there are large numbers of cells apparently of endothelial origin, rounded, oval, or polygonal, with abundant protoplasm and large nucleus. The whole of the protoplasm is packed with bacilli, and a few organisms are also found lying free (Fig. 3, Plate VII).

Giant cells are numerous and occasionally of enormous size, the transverse diameter reaching 70—80μ, and containing many nuclei which may be situated either in the centre or at the periphery of the cell. They are sometimes vacuolated. The protoplasm of these cells is almost entirely occupied by masses of bacilli. They form beautiful objects either in smear preparations of glands or in sections (Fig. 2, Plate VII). Their mode of origin is difficult to determine just as in the case of lepra. It seems probable that they are greatly enlarged endothelial cells in which multiplication of the nuclei has taken place. The view, however, may be held that they are small lymphatic vessels which have been blocked by bacilli and that the nuclei are those of the endothelium originally lining the channel. The continuity of the cell outline is retained till a great size is reached, but in certain cases the cells seem to have become disintegrated from the overgrowth of bacilli.

Other Lesions.

When the cervical lymphatic glands were greatly enlarged an invasion of the neighbouring structures such as the submaxillary salivary glands was observed. Visceral lesions are rare—only in one case in a naturally infected animal has a small necrotic area containing the bacilli been found in the liver. The bone-marrow was found to be invaded.

An acid-fast bacillus was found in two cases in the nasal secretions, probably identical with that causing the lesions. This is interesting in relation to Stricker's (1899) observation of the frequency of the *Bacillus leprae* in the nasal mucus.

In one rat the loss of several toes was observed, and in others part of the tail had disappeared. As the writer has observed that bacilli

sometimes invade the nerve-trunks these losses may be connected with nerve changes. The lesions may, however, have been the result of trauma and not of disease.

The health of the diseased animals is sometimes seriously affected, and emaciation is a marked feature. Some of the animals were captured in broad daylight wandering outside their holes in a dazed condition.

The disease is evidently of wide distribution since it has been observed in Odessa, Berlin, and England. Six of the seven well-marked examples of the disease which have been examined were obtained within a few miles of Elstree, Herts.; the seventh was found in Norfolk.

The Bacilli.

The bacilli are straight or bent rods with round, or more rarely pointed ends, about 0·5 μ broad and for the most part from 3 to 5 μ in length, though sometimes longer forms are observed. They may have a beaded appearance, the beads being few and large, and causing a bulging of the rod. The bacilli are strongly acid- and alcohol-fast. They stain readily with carbol-fuchsin, and resist the action of 25 % sulphuric acid or 3 % hydrochloric acid in alcohol, or 2 % anilin-hydrochloride—followed by alcohol. They stain by Gram's and Claudius' methods.

An interesting point about the staining of the bacillus which it has in common with that of leprosy is that if mounted in even slightly acid Canada balsam it gradually loses its bright red colour.

Cultivation experiments.

All attempts at cultivation of the bacillus in its acid-fast form have entirely failed. A large number of tubes containing all the ordinary laboratory media have been inoculated and kept under aerobic and anaerobic conditions but no growth took place. Special media have also been tried, such as a medium made from rat's flesh, and a medium with the addition of rat's blood-serum, but these have also failed to give a growth of the bacillus. Many pieces of tissues swarming with bacilli which have been left for over a year on appropriate media still show the bacilli staining well but no growth has taken place. As Rabinowitsch has pointed out, this makes it improbable that the bacillus is one of the known earth or dung bacilli, because these are easily cultivated and appear to be innocuous to rats. The writer has succeeded in obtaining from two of the affected rats cultivations of a diphtheroid bacillus.

This observation is of interest because a diphtheroid bacillus has been isolated by Babes and a number of observers from cases of lepra.

In his later publications Babes (1899) states that he has isolated this organism from twelve cases. Spronck (1898) isolated the same organism and found that it was agglutinated by the blood-serum of leprous patients, though not more so in certain cases than by the serum of non-leprous persons. As is remarked by Babes, a positive agglutination test would not prove the identity of this diphtheroid bacillus with the lepra bacillus, since if the bacillus is frequently associated with the acid-fast bacillus in cases of lepra it would naturally give rise to an agglutinating serum in these patients. The identity of the two bacilli would be demonstrated in a satisfactory manner only (1) if the diphtheroid bacillus could be so cultivated as to become acid-fast, (2) if it were demonstrated to have specific properties in relation to lepra such as the production of a specific tuberculin-like reaction, or (3) if it was found capable of producing lepra. Babes has carried out experiments in certain of these directions without, however, obtaining conclusive results.

Levy (1897 and 1899), Czaplewski (1898), Barannikow (1899), Teich (1899), and Kedrowski (1901) have all obtained cultures of bacilli resembling those of Babes. Kedrowski (1904) believes that he has obtained lesions resembling those of lepra by injecting his bacillus into rabbits.

The diphtheroid bacillus, isolated by the writer from rats, resembles the organism isolated by these workers, but its significance is open to the same criticism since, so far, it has been found impossible to convert it into the acid-fast one. There was no evidence of the acquisition of acid-fast properties by the diphtheroid bacillus after a sojourn of three months in the peritoneal cavity of the rat.

The only observation that makes it possible that the two organisms are in any way related will be found under the head of agglutination. A probable explanation of the presence of diphtheroid bacilli in lepra, and it may be also in the rat disease, is the fact that diphtheroid bacilli appear frequently to have their habitat in sebaceous glands, and in a weakened state of the skin they may be able to penetrate it and grow in the tissue.

Brief description of the main characters of the diphtheroid bacillus which was isolated from two of the diseased rats.

Morphology. In young cultures (24 hours old, on agar, or blood serum) it has the form of a small diplo-bacillus, or is sometimes very

like a Hofmann's bacillus. On the second or third day it becomes longer and has all the appearances of a typical diphtheria bacillus with segmentation, club-shaped ends, etc., and later it may form short, much segmented and branching filaments. In a two days' old culture, the club-shaped forms become very large, much segmented, and recall those of the *B. xerosis.*

Cultures. On solidified blood serum the growth is indistinguishable from that of the Klebs-Loeffler bacillus. On neutral agar and glycerine agar the growth is slower, the colonies are more delicate and transparent than those of the ordinary diphtheria bacillus. On neutral and alkaline broth it forms no pellicle, but grows in the form of clumps along the test-tube wall, or as a flocculent deposit at the bottom of the test-tube. In glucose broth the growth is granular but not so abundant as that of the diphtheria bacillus. It forms acid in 24 hours, but not so strongly as the diphtheria bacillus. It forms no visible growth on potato.

Pathogenicity. The diphtheroid bacillus has a feeble pathogenic action on young rats. Three out of four young rats that received 2 c.c. of a broth culture a week old died within a week. Old rats proved resistant.

Agglutination tests.

No great importance can be attached to these agglutination tests in view of the absence of specificity which some observers have demonstrated in regard to the agglutination of the acid-fast group of bacilli. The tests are of a preliminary character and are included in this paper merely as an indication of a line of research which it was thought might throw some light on the relationship of the organisms.

Preparation of bacterial suspension. A piece of tissue, very rich in the acid-fast bacillus of the rat, was removed from the methylated spirit in which it had been preserved, allowed to dry, and ground up in a mortar with glass powder in normal salt solution. After standing for some time it was found that nearly all the fine pieces of tissue had sedimented, and that the supernatant opalescent fluid consisted almost entirely of acid-fast bacilli. The fluid was brought to a degree of opacity suitable for carrying out naked eye tests in Wright's agglutination tubes which were used on account of a very small quantity of serum being available. The bacterial suspension remained almost unaltered for 24 hours.

TABLE I. *Result in three hours.*

TEST 1.

Dilution	Normal Serum	Leprous Serum
1 : 2	Nil	Large sediment
1 : 4	,,	,, ,,
1 : 8	,,	,, ,,
1 : 16	,,	,, ,,
1 : 32	,,	Distinct sediment
1 : 64	,,	Trace

TABLE II. *Result in sixteen hours.*

TEST 2.

Fresh Dilutions	Normal Serum	Leprous Serum
1 : 2	Nil	Large sediment
1 : 10	,,	,, ,,
1 : 20	,,	,, ,,
1 : 40	,,	Distinct sediment
1 : 50	,,	Trace
1 : 60	,,	Nil

TABLE III. *Agglutination of diphtheroid bacillus emulsion prepared from agar culture of three days' growth by means of shaking with glass beads.*

Serum	1 : 2	1 : 10	1 : 20	1 : 30	1 : 40
Normal (human)	0	0	0	0	0
Human (tubercular case)	0	0	0	0	0
Lepra serum	+	+	+	+	0
Normal rat	0	0	0	0	0
Rat (inoculated with acid-fast bacilli)	+	+	0	0	0
Rat 2 (ditto)	+	+	0	0	0

In these tests:—

(1) the serum from a case of human leprosy agglutinated the acid-fast bacillus from the rat; normal human serum had no agglutinating power;

(2) the serum of rats inoculated with the acid-fast bacilli agglutinated the diphtheroid bacillus; normal rat's serum did not agglutinate the bacillus;

(3) normal human serum and the serum from a tuberculous patient failed to agglutinate the diphtheroid bacillus, whereas the serum from a case of leprosy had distinct agglutinating properties.

Note. The serum from only one normal, one tuberculous, and one leprous patient, was used in these tests. I wish here to express my thanks to Dr Lie of Bergen for his kindness in supplying some blood from a case of leprosy.

Inoculation Experiments.

I. Rats.

Thirty black and white rats have been inoculated with material consisting of emulsions made from the infiltrated skin and enlarged lymphatic glands obtained from the naturally, or experimentally, infected animals. A considerable number of these died of intercurrent disease without obvious signs of having been infected with the acid-fast bacillus, though in many of them, even in the absence of naked-eye changes, the organism could be demonstrated to be present in the peritoneum after two or three months. Nine animals, however, showed marked lesions, evidently produced by a multiplication and invasion of the acid-fast bacillus, and in some of these the lesions were of a very striking character.

In three cases the infection was carried on successfully from the naturally infected animal to an experimental animal and from the experimentally inoculated rat to a third rat: the lesions in the two generations of experimentally infected animals were similar.

Course of the Disease in the Inoculated Rats.

The disease runs a very slow course in the inoculated animals. In one case a rat died about one year after inoculation with well-marked evidence of a progressive invasion of the bacillus. In others well-marked lesions developed and death occurred within six months of inoculation. There was no definite evidence of increase of virulence in the third generation.

Pathology.

Subcutaneous Inoculation was in several instances followed by the production of a local lesion but this was not a constant result. The lesion consisted of a nodule containing a semi-caseous-like substance resembling the material which was present in the skin infiltration of the naturally infected animals. The acid-fast bacilli were very numerous in these local lesions. The lymphatic glands in such cases, even at a distance from the lesion, were sometimes invaded by the bacilli and showed the changes already described. For example, in one case where the local lesion was situated in the groin the axillary lymphatic glands were infected.

Intraperitoneal Inoculation. The most marked lesions resulted from intraperitoneal injections. In these the epiploön presented a

striking appearance. It was frequently infiltrated with grey opaque looking masses of material which on microscopical examination proved to be masses of cells containing bacilli. The peritoneum was in some cases thickened, and pale yellow patches of infiltration occurred on both the visceral and parietal surfaces.

Liver. As a rule, even when the infection was well-marked, the liver showed nothing abnormal or the capsule only was involved. In one case, however, the organ was beset with small, pale, yellow, sharply circumscribed areas to such an extent that about a third of the liver substance seemed to be destroyed. A second case showed the condition in a less advanced stage. The areas under the microscope were seen to be sharply circumscribed and surrounded by flattened liver cells. The process was very extensive close to the peritoneum and seemed to extend along the Glisson's capsule. When stained by Ziehl-Neelsen's method the appearance presented under a low power is shown in Plate VII, Fig. 1, which is typical of what is found throughout the liver. The bulk of each nodule appears to be composed of masses of acid-fast bacilli. At the periphery these are mostly arranged in round masses as if lying in cells or cell remains. Some of these round masses are very large and so tightly packed with bacilli that they appear almost homogeneous. In the centre of the nodule the cellular appearance may be less marked, many of the bacilli appearing to be free. Round some of the nodules the capillaries are greatly dilated, and in the endothelium of these there are groups of acid-fast bacilli.

Spleen. The capsule was thickened and contained bacilli in several cases, but only in one was the organ itself markedly involved. This occurred in the case in which the liver was most affected.

Kidneys, Bladder, and Genital Organs. In several instances the capsules of these organs were thickened and infiltrated with bacilli but so far as could be found no extension of the process to the organ itself had occurred. In the case of the testicle and epididymis groups of bacilli were found lying in the connective tissue framework of the organ.

Thorax. In three of the inoculated animals a very striking appearance was found on opening the thorax. A tumour-like mass occupied the anterior mediastinum, and the posterior surface of the sternum was covered with an infiltrating material which in one case reached a thickness of a quarter of an inch. The pale yellow tumour-like mass occupied a considerable part of the thorax and was moulded over the pericardium and apices of the lungs. On microscopical

examination only small areas of lymphatic gland tissue were visible, the bulk of the mass consisting of the acid-fast bacilli. This tendency, especially in rodents, for the anterior mediastinal glands to become infected from the peritoneum has been frequently observed.

Lungs. Lesions of these organs were not common, but in two cases a few small nodules, the size of millet seed, were observed. These appeared to originate from the lymphatic vessels lying beside the small bronchi and vessels.

The *Pericardium* and *Epicardium* were secondarily invaded in these cases with retrosternal tumours.

Myocardium. A small nodule containing the bacilli was in one case found in the myocardium.

Blood. On several occasions bacilli were seen lying within the blood-vessels.

The Central Nervous System. In two cases the bacilli were looked for in the brain and spinal cord but were not found.

Nerve-trunks. Bacilli were found to have invaded these both in the experimental animals and in those naturally infected.

II. Inoculation of Animals other than Rats.

Guinea-pigs. Thirteen animals were inoculated; of these only one developed a small local subcutaneous lesion which contained the acid-fast bacilli. None of the others showed any effect.

Rabbits. Six animals inoculated; showed no effect.

Mice. Ten animals inoculated. Six of these died, but without showing any lesions which could be attributed to the acid-fast bacillus.

Monkey (*Macacus rhesus*). After 18 months showed neither local nor general signs of infection.

These results show that we are not dealing with Tubercle bacilli, especially as in some of the guinea-pigs and rabbits very large doses were given.

Remarks and Conclusions.

One of the chief features common to this disease in the rat and to lepra in the human subject, namely the presence of the bacilli in such large numbers within the cells, has been regarded by Babes as the evidence of a form of symbiosis between the cells and the bacilli. The bacilli invade the cells and live within them without interfering with their vital functions.

It seems probable, however, that we are both here and in leprosy dealing with the phagocytosis of a relentlessly invading germ of great resistance, which, notwithstanding the fact that it finds itself in a suitable medium, has little power of producing toxic substances. In the case of tubercle, after a few bacilli have been taken into a cell, so much toxin is secreted by the bacilli that necrosis and death of the cell occurs. Here, on the other hand, the bacilli when taken into the cells are able to continue their growth and having only feeble toxigenic properties they have relatively little deleterious action on the cells, which maintain their vitality and for long continue to struggle against the invaders. The slowness of the struggle may possibly account for the enormous size of the giant cells.

It would be out of place here to go into the literature of the attempts and failures to inoculate animals with the Lepra bacillus; one may, however, refer to the work of Iwanow (1902), who, working in Metchnikoff's laboratory, succeeded in obtaining results in guinea-pigs which he regarded as indicating that a certain multiplication of bacilli may have taken place. The appearances which he observed in the epiploön of the guinea-pig resemble those observed in the slighter form of invasion in the case of the rat disease. It would be of interest to know whether the rat has been used for inoculation experiments with leprous material. So far as could be learned from a brief review of the subject this does not appear to have been the case [Wolters (1893); Finger (1901), and Hansen (1902)].

The features of resemblance between these two diseases are so strong that they point to a close relation between the associated bacilli.

The consideration that man and the rat are fellow victims of almost equal susceptibility to other disease germs such as the plague bacillus, might even suggest the possibility that in lepra and in this rat disease we are dealing with the same micro-organism affecting two species.

There is no intention here, however, in the present state of our knowledge to uphold such a hypothesis, which is obviously rendered improbable from epidemiological considerations alone.

As has been indicated in discussing the interpretation of the appearance in the infected animals, many of the pathological features of resemblance probably depend on the slow invasion of, and the defence offered by, the animal's tissues against a highly resistant organism which is not markedly toxigenic.

CONCLUSIONS.

1. This disease of the rat has a remarkable resemblance, both in its pathological, anatomical, and in its bacteriological features to Lepra in the human subject.

2. All attempts at cultivation of the acid-fast bacillus have failed.

3. A diphtheroid bacillus has been isolated and cultivated from two cases of the disease, but so far attempts to convert this into an acid-fast form have been unsuccessful.

4. The experiments on agglutination having been of a preliminary character do not permit as yet of any definite conclusions. Nevertheless the bacillus was agglutinated by the serum of a leprous patient.

5. The disease could be conveyed to other rats (but not to rabbits, guinea-pigs, mice or a monkey) by subcutaneous and by intraperitoneal injection of emulsions of the bacilli from infected tissues.

6. A large number of the rats inoculated succumb to intercurrent diseases but a certain number develop well-marked characteristic lesions which can be again reproduced by reinoculation.

7. The disease in these artificially infected animals runs a very slow course, which may extend to about one year.

EXPLANATION OF PLATES VI AND VII.

Plate VI. Fig. 1. Photograph of rat (*Mus decumanus*) showing the appearances presented in a case of natural infection with the acid-fast bacillus. Note the patches of alopecia, two of which show small ulcers.

Fig. 2. Photograph showing a dissection of the enlarged cervical and axillary lymphatic glands in a case of natural infection of the rat with the acid-fast bacillus.

Plate VII. Fig. 1. Drawing of the microscopical appearances presented by a section of the liver of a white rat which had been inoculated intraperitoneally six months before death. The red colour of the affected areas in the liver is due to the presence of masses of the acid-fast bacillus. Ziehl-Neelsen's solution, 3 % hydrochloric acid-alcohol, aqueous methylene blue. × 100.

Fig. 2. Drawing of a giant cell containing bacilli. From a smear preparation of the axillary gland of a white rat which died eleven months after subcutaneous inoculation. Ziehl-Neelsen's solution, 25 % sulphuric acid, aqueous methylene blue. Under oil immersion lens. × 825.

Fig. 3. Drawing of the microscopical appearances of a section of an infected lymphatic gland. Shows the cells of endothelial origin packed with acid-fast bacilli. Ziehl-Neelsen's solution, 3 % hydrochloric acid-alcohol, aqueous methylene blue. × 825.

Fig. 4. Same cell as seen in Fig. 2. × 100.

Fig. 5. Drawing of the microscopical appearances of a section through the affected skin of a rat suffering from the naturally acquired disease due to the acid-fast bacillus. Most of the red colour below the epithelium is due to the presence of large numbers of bacilli. Stained with Ziehl-Neelsen's solution. 25 % sulphuric acid, aqueous methylene blue. × 100.

The drawings are by Mr M. H. Lapidge.

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ON THE TREATMENT OF CANCER WITH RADIUM BROMIDE.

BY H. G. PLIMMER, M.R.C.S. ENG., F.L.S.

IN November last Lord Iveagh generously placed at my disposal some radium bromide, with the request that I would investigate its effects upon cancer. The staff of the Cancer Hospital allowed me to make use of the following cases, so that I was enabled to follow the effects of the action of radium more or less completely on 17 cases which I have tabulated in a form giving a general view of the cases and the extent of the treatment. The amount of radium bromide used for these cases was 30 milligrammes and it was inclosed in a vulcanite capsule covered with talc. It was made by the Braunschweig Chinin Fabrik.

It will be noticed that this series of cases consists entirely of carcinoma, many of the cases being in a very advanced condition. The diagnosis was confirmed in all of them either by the examination of the primary growth or by the excision of a nodule. The individual exposures varied from 40 minutes to five minutes; and the capsule containing the radium was placed directly on the nodules or lumps, the only thing intervening between the radium and the skin being the talc top of the capsule. I left the cases for a month after the exposures and then examined them to see in what condition they were. The effects in all the cases were negative, either no change or an increase in the size of the nodules. Cases 3, 14, and 17 were all about to slough when they came for treatment, and there is nothing whatever to indicate that the sloughing was in any way hastened by the treatment. The effect on the skin varied in the different cases; an exposure of ten minutes was generally sufficient to produce blistering, and then scabbing, and under the scabs there was found a very slowly-healing ulcerated surface. The radium had apparently no effect with regard

TABLE OF CASES OF CANCER

No.	Sex.	Age.	Nature of cancer.	Total time of application of radium.	
1	F.	53	Three nodules on the right chest, secondary to cancer of the breast.	20 minutes.	
2	F.	42	Two nodules on the left chest, secondary to cancer of the breast.	10 minutes.	
3	M.	40	Sloughing epithelioma of the lip.	10 minutes.	
4	F.	28	Multiple nodules on the chest, secondary to cancer of the breast, and a gland above the clavicle.	2 hours 50 minutes to nodules and 45 minutes to gland.	
5	F.	40	Ulcerated contracting scirrhus of the breast.	25 minutes.	
6	F.	44	Recurrent growth of the breast in and about the scar.	11 hours 55 minutes.	
7	F.	44	Gland deep in the axilla.	36 minutes.	
8	F.	80	Moveable lump in the right breast about three inches by two inches.	14 hours 25 minutes.	
9	F.	64	Diffuse mass of secondary ulcerated cancer in the right axilla.	1 hour 30 minutes.	
10	F.	54	Cicatrix adherent to the ribs, with small nodules in it.	3 hours 45 minutes	
11	F.	53	Diffuse mass of secondary cancer in the right axilla.	2 hours 35 minutes.	
12	F.	63	Small nodules beneath the skin between a cicatrix and the clavicle on right chest.	1 hour 20 minutes.	
13	F.	60	Secondary growth in the axilla and gland above the clavicle.	3 hours 15 minutes.	
14	M.	53	Fungating mass below the jaw, secondary to epithelioma of the lip.	15 minutes.	
15	F.	52	Two very small subcutaneous secondary nodules near cicatrix of the breast.	3 hours 10 minutes.	
16	F.	60	Secondary glands in the axilla.	1 hour 30 minutes.	
17	F.	48	Large mass of secondary growth over the sternum, about to slough.	1 hour 10 minutes.	

est and est time pplica-ion.	Skin.	Remarks.
inutes.	Reddened.	The patient died on Dec. 31st. A nodule was removed on Nov. 25th to see what effect had been produced.
nutes to nodule.	,,	Nodules have ulcerated and the patient is considerably worse.
inutes.	Broke down two days after the last application.	This was a raised nodule in a diffuse epithelioma of the lip which was soft at the time of application.
inutes; inutes.	Blistered and scabbed.	The gland at first seemed softer but did not diminish in size. A nodule was removed for diagnosis.
inutes.	Reddened.	The patient died on Jan. 23rd.
inutes; inutes.	Blistered and scabbed.	The nodules increased rapidly in size.
inutes; inutes.	Reddened.	No change.
inutes; inutes.	Blistered and scabbed, at first blackish in colour, later white and scaly.	This patient showed no sign of improvement in the primary growth and an axillary gland enlarged to a good size during treatment.
inutes.	Very thin and red before treatment.	The patient died on Jan. 11th.
inutes; inutes.	Blistered early.	No change.
inutes; inutes.	Very red and angry before treatment.	The patient thought she could move her arm better but there was no change in the growth.
inutes.	Blistered and scabbed.	No change.
inutes; inutes.	,,	The patient thought she could move the arm better but no change in the growth was effected.
minutes.	Sloughing.	The mass sloughed out a few days after.
inutes; inutes.	Blistered.	There was no change in the nodules and a new one appeared during treatment.
inutes.	,,	No change.
inutes; inutes.	Reddened.	The growth began to slough.

either to causing or to relieving pain and the irritation of the blisters was slight.

From three of the above patients who died some nodules which had been treated were excised and subjected to a careful microscopical examination. In all these cases no change was found either in the cancer cells or in the fibrous tissue and none were degenerated. There appeared to be an increase in the amount of the subcutaneous round-celled exudation which was greater in amount in those nodules which had been more recently treated, but the irritation of the skin would sufficiently account for this. On the other hand, Dr. Apolant, writing from Professor Ehrlich's laboratory, records[1] some very satisfactory and striking results obtained by treating very rapidly growing nodules of inoculated cancer in mice with radium. The nodules decreased remarkably in size, but it was apparently difficult to get rid of the last bit of the nodule, probably on account of fibrous changes. These results are much the same as those obtained by Professor Exner in Vienna in a case of melanotic sarcoma with multiple rapidly growing nodules in the skin. It appears as if the emanations from radium can only act upon young and rapidly growing cells and that older cells, especially if surrounded by fibrous tissue, are less and less easily affected, and if there be an excess of fibrous tissue the cells are not at all affected.

Lister Institute of Preventive Medicine, S.W.

[1] Deutsche Medicinische Wochenschrift.

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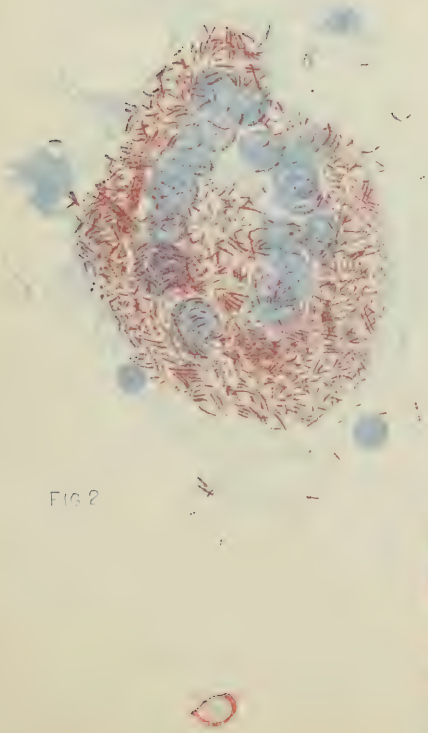
Fig. 1.

Fig. 2.



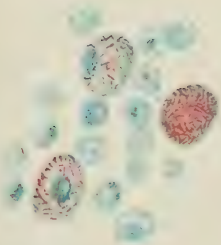
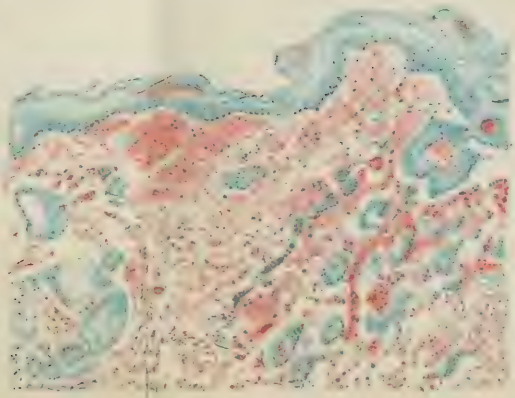


FIG 2



[*From the* PROCEEDINGS OF THE ROYAL SOCIETY, Vol. 74.]

"Note on the Effects produced on Rats by the Trypanosomata of Gambia Fever and Sleeping Sickness." By H. G. PLIMMER, F.L.S. Communicated by Dr. C. J. MARTIN, F.R.S. Received December 1, 1904,—Read January 19, 1905.

(From the Laboratories of the Lister Institute of Preventive Medicine.)

The following observations are of interest in connection with the view that Gambia Fever may be the early stage of the disease which ends as Sleeping Sickness, which is held by some: and with the question of the causal relationship of the Trypanosoma found in Sleeping Sickness to the disease.

The Trypanosomata used in the experiments mentioned below were given to me by Col. Bruce, F.R.S.; that of Gambia Fever having been taken from a monkey inoculated in Africa from a native boy suffering from Gambia Fever, and that of Sleeping Sickness from a monkey inoculated in Africa from a case of this disease.

Gambia Fever.

Fourteen rats were inoculated with the Trypanosoma of Gambia Fever at intervals between December 11, 1903, when I received the first rat from Col. Bruce, and August 24, 1904, all of which are dead. In each case the Trypanosomata were present in the blood, the first appearance of them there being about four weeks after inoculation: and towards the end of the disease they were present in large numbers. The average time between inoculation and death was two months and twelve days. In each case the spleen was enormously enlarged; there was considerable hyperplasia of the follicles, and the pulp was congested in varying degrees. There was also marked hyperplasia of the lymphatic glands, especially of those of the mesenteric and mediastinal areas. There was congestion of the liver, with cloudy swelling of its epithelium, and the same condition in a lesser degree was found in the kidney. In no case was there any paralysis, or any nervous symptoms, except that just before death the animals became heavy and apathetic. *Post mortem* the capillaries of the brain contained large numbers of the organisms.

Sleeping Sickness.

Three rats were inoculated on December 1, 1903, from a monkey which had been brought home by Col. Bruce from Africa after inoculation with cerebro-spinal fluid from a case of Sleeping Sickness. At no time were any Trypanosomata found in the blood of these rats, even after it had been centrifugalised *post mortem* One rat became

paralysed in both hind legs on May 12, 1904, and died on May 23; the second became paralysed similarly on August 2, and died on August 30; and the third became paralysed on August 28, and died on September 8. On *post mortem* examination no naked-eye lesions of any organ were found, and the spleens were not enlarged. The blood was citrated and centrifugalised, and the organs were mashed and washed with normal salt solution and centrifugalised, but in no case were any Trypanosomata found. Portions of the extracts of liver, spleen, and spinal cord were injected into other rats, but up to the present these show no sign of illness. In the mashed spinal cord of each of these rats the characteristic Trypanosomata were found in small numbers, but none were found in the brains which were examined in the same way. The three rats were of the same kind, black and white; and the youngest of the three died first and the eldest last.

---

(1) These experiments go to show that the two diseases—Gambia Fever and Sleeping Sickness—from which the organisms were obtained, are distinct; the duration of the diseases, the symptoms, and the *post mortem* appearances being quite different.

It is evident that these two organisms are quite separate and distinct, as their different effects on similar animals indicates; moreover, they are also morphologically distinct when grown in similar animals, that of Gambia Fever being longer, generally larger, and more easily stainable than the stumpy, large-vacuoled, badly staining Trypanosoma of Sleeping Sickness.

The fact of the clinical observation that Gambia Fever not infrequently appears to terminate with all the symptoms of Sleeping Sickness may quite possibly be explained by a double infection. For, in both rats and monkeys the one Trypanosoma does not interfere with the other, but the more active organism—that of Gambia Fever in the cases of rats and monkeys—kills in about the same time, whether inoculated before, with, or after that of Sleeping Sickness.

(2) There can be no question, from the above experiments, of the susceptibility of the rat to the Trypanosoma of Sleeping Sickness.

(3) These experiments show that the inoculation of the Trypanosoma of Sleeping Sickness into rats gives rise to no obvious symptoms for many months, nor are Trypanosomata discoverable in the blood by microscopic examination. But, after a period of from six to nine months paraplegia occurs, leading to the death of the animal; and *post mortem* the organisms are found only in the spinal cord. The organisms are thus in rats, as sometimes in man, entirely confined to the nervous system; whereas in monkeys they are, in my experience, always generalised at some period of the disease.

Addendum, January 19, 1905.—The three rats inoculated on August 30, 1904, with extract of spinal cord have become paraplegic: one on December 14, 1904, one on January 2, 1905, and the third on January 11, 1905. No Trypanosomata have been seen in the blood of these animals, and, apart from paralysis of the hind limbs, they show no signs of ill health.

The spinal cord shows amœboid forms of the Trypanosomata in its tissue, and a considerable cellular exudation around the vessels; this lesion Dr. Mott found to be characteristic of cases of human Sleeping Sickness, and it is not found in monkeys, which have been the animals principally used in experimental work on this disease.

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ON A DYSENTERY TOXIN AND ANTITOXIN.

By CHARLES TODD, M.D. (Cantab.),

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I. *Introduction.*

One of the most striking points in the pathology of Bacterial Dysentery is the fact that while the bacilli are found regularly in the mesenteric glands, they are not found in the spleen or other organs, and

do not occur in the blood, urine, or milk. In this respect Lentz (p. 320) points out that the disease differs markedly from the septicaemic diseases, such as typhoid, and that it must be regarded as a local infection of the intestinal mucous membrane and corresponding lymphatic glands by the bacillus, the toxin alone passing into the circulation and giving rise to the typical clinical picture, which, as in the case of cholera, gives the impression of a severe poisoning or toxaemia.

The results of experiments on animals with the *Bacillus dysenteriae* (Shiga) bear out this view, as the pathological changes which result from the injection of the living bacillus may be produced equally well by the use of dead cultures; showing that these changes are brought about by some toxic body, and not by any vital action of the bacillus.

The following experiments, which were commenced at the end of 1902, were undertaken with the view of investigating the nature of this toxic body, and in the hope of obtaining an anti-body for it. A preliminary note of the earlier results was published by me in the *British Medical Journal* in December, 1903. At the time the experiments were begun the only reference which could be found in the literature to a soluble dysentery toxin was the statement by Lentz (p. 328) in the article on dysentery in Kolle and Wasserman's *Handbuch der pathogenen Microorganismen*, "that the filtrates of dysentery cultures are toxic." Subsequently, however, Gay (xi. 1902) showed that vaccines prepared by killing the dysentery bacillus by heat and tricresol, or by tricresol alone, undergo on keeping an increase in toxicity accompanied by a shortening of the time necessary to produce death. This is attributed to a process of breaking up of the bacterial bodies.

Conradi (1903) also stated that he was able by a process of "aseptic autolysis" to extract from the dysentery bacillus a soluble toxin, and shortly after this Neisser and Shiga were able to obtain a similar toxin by suspending the bacillus in saline, killing by exposure to heat, keeping the suspension for two days at 37° C., and then filtering. Finally Rosenthal also found that by growing the bacillus in Martin's alkaline broth highly toxic filtrates are formed.

In the present experiments the following bacilli were used:

1. *B. dysenteriae* Shiga.
2. „ Kruse (obtained from Král).
3. „ Flexner (obtained from Král).
4. „ Flexner (Adult Dysentery, Philippines).

5. *B. dysenteriae* Duval (Summer Diarrhoea, Baltimore).
6. „ Duval (Summer Diarrhoea, New York).
7. „ Eyre No. 1 (Asylum Dysentery, England).
8. „ Eyre No. 2 „ „
9. „ Eyre No. 3 „ „

These cultures, with the exception of Nos. 2 and 3, which were obtained from Král of Prague, were very kindly sent me by Professor Flexner and Drs Shiga, Duval, and Eyre.

The toxigenic power of each of these strains was tried, but for most of the experiments Kruse's bacillus was employed, and where the strain of the bacillus is not specified this bacillus is referred to.

II. *Production and Characters of the Toxin.*

The filtrates from ordinary broth cultures of the bacillus are only slightly toxic for animals, but it was found that by using a more alkaline medium much more highly toxic filtrates could be obtained.

For the production of the toxin the ordinary alkaline broth as employed for the production of diphtheria toxin (that is, broth which has been made just alkaline to litmus and then has had added to it 7 c.c. of normal caustic soda per litre) was planted with the bacillus; grown for a varying period at 36° C., and then filtered through a Pasteur-Chamberland filter and the filtrate tested on rabbits by intravenous injection. At first the cultures were grown for ten days before filtration. The following table shows some of the results:

TABLE I. *Toxins obtained by growing the Bacillus for Ten Days in Alkaline Broth.*

No. of toxin	Bacillus	M.L.D. for large rabbits
No. 1	Kruse	1 c.c.*
„ 2	„	„
„ 3	„	„
„ 4	„	„
„ 5	„	„
„ 6	Shiga	„

* In the tests the minimal lethal dose (M.L.D.) was only approximately determined, that is, the toxin killed at 1 c.c. and not at 0·5 c.c.

Later it was found that by growing the cultures for a longer period much more toxic filtrates resulted, as shown in the following:

TABLE II.

No. of toxin	Bacillus	M.L.D. for large rabbits
K. 8	Kruse	0·10 c.c.
K. 9	,,	0·10 ,,
K. 11	,,	0·10 ,,
K. 15	,,	0·10 ,,

The best results, as regards the toxicity of the filtrates, appears to be reached in about a month to six weeks, and after this the toxicity begins to fall. The filtrate from a three months' old culture was found to be incapable of killing large rabbits at 0·5 c.c. though this dose gave rise to paralysis and diarrhoea. Experiments were made with a bouillon containing twice the amount of alkali but this did not give any increase of toxicity. The toxin in the filtrate is moderately stable—a toxin which had been kept at room temperature for 4½ months being still highly toxic—and is not destroyed by heating at 70° C. for 1 hour, though exposure to 80° C. for an hour seems to entirely destroy it. It is precipitated by ammonium sulphate; thus a filtrate which killed rabbits at 0·1 c.c. intravenously was precipitated with ammonium sulphate, the precipitate dissolved in distilled water and again precipitated. After drying *in vacuo* over sulphuric acid the final precipitate killed large rabbits at 0·002 grammes as shown in the following table:

TABLE III. *Precipitated Toxin (K. 12) tested on Rabbits intravenously.*

Dose in grammes	Weight of rabbit in grammes	Result
0·10	1050	Dead (24 hours)
0·01	880	Dead (3 days)
0·004	1250	Dead (2 days)
0·002	1480	Dead (4 days)
0·002	1700	Recovered after paralysis
0·001	1600	Lived, no paralysis
0·001	1600	Lived, slight paralysis

The results of the intravenous injection of a lethal dose of the toxin in a rabbit are practically the same as when the living, or dead bacillus is used. After a latent period, varying from one to three or four days, according to the amount of toxin injected, severe diarrhoea sets in with paralysis of the limbs, beginning sometimes in the fore, and sometimes in the hind limbs, but ultimately affecting both, as well as the muscles of the trunk and neck. The animal rapidly loses weight, and death occurs in from one to four or five days. *Post mortem* the lesions

resemble those following the injection of the bacillus. There is marked congestion of the large intestine, which may show small haemorrhages and contains a slimy fluid. The lungs show patches of congestion and often small haemorrhages, the other viscera do not, as a rule, exhibit any marked naked-eye changes.

In the rabbit the toxin acts most energetically when given intravenously, but the same result follows subcutaneous, intramuscular or intraperitoneal injections if large doses are employed.

The variation in the susceptibility of different animals to the toxin is very striking. The rabbit and horse are highly sensitive, the latter particularly so, while the guinea-pig, rat, mouse and monkey are hardly affected; thus, 0·1 g. of the precipitated toxin K. 12 (v. Table III), *i.e.*, 50 minimal lethal doses for a large rabbit, when injected subcutaneously in a guinea-pig caused no symptoms beyond transient local swelling and loss of weight.

III. *Immunisation of Animals.*

Attempts to produce an antitoxin by immunisation of rabbits were entirely negative, the serum of an animal which had received a considerable amount of toxin and which agglutinated the bacillus at 1 in 100 showing absolutely no antitoxic power. A goat was immunised with increasing doses of the toxin, beginning with 0·1 c.c. and rising to 200 c.c. The serum of this animal, taken on the 13th day after the last injection, was tested against the toxin on rabbits and showed distinct, though slight, antitoxic power. The animal was bled again on the 33rd day after the last injection and the serum from this bleeding was found to be much more highly antitoxic. This result possibly explained the want of success in the case of the rabbit when the bleedings had been carried out on the 9th day after the last injection and probably before the antitoxic power had reached its maximum. A horse was then immunised with the toxin. The details of this immunisation are interesting as showing the great susceptibility of this animal to the toxin. The toxin used was a very weak one, 1 c.c. being required to kill a rabbit when given intravenously.

The immunisation was begun in December, 1902, by giving 0·5 c.c. of this toxin subcutaneously and injections of increasing quantities were made every 3rd day for a period of 6 weeks when a dose of 150 c.c. was reached. After a dose of 2 c.c. of the toxin the horse had a rise of temperature (102·2° F.) the same evening, and the next day had very pronounced diarrhoea, and was off his feed. Later on, after a dose of

8 c.c., diarrhoea again appeared, but with these exceptions the animal appeared to stand the immunisation well. On the 5th day after the final injection of 150 c.c., however, complete paralysis of the hind legs occurred and the animal had to be killed.

A second horse was then immunised, beginning with a dose of 0·1 c.c. of a toxin of the same strength and very cautiously increasing the doses, which were repeated at intervals of not less than a week. In this case the immunisation was quite satisfactory, no symptoms being shown beyond the transient local swelling and rise of temperature following the injection. Finally, a dose of 400 c.c. of a toxin which killed a large rabbit at 0·1 c.c. was reached, after which the animal was allowed to rest for a month and then bled.

For purposes of comparison a third horse was immunised, and in this case the bacillus alone, *i.e.* without its soluble toxic products, was used. Kruse's bacillus was grown for twenty hours on neutral agar and the growth emulsified with saline. At the beginning of the immunisation this emulsion was heated for half-an-hour at 70° C., and given subcutaneously, beginning with a dose corresponding to one-tenth of 1 tube and gradually rising to 32 tubes. After this an emulsion of the living bacillus was given intravenously until a dose of 10 agar tubes of living culture was reached. One month after the last injection the horse was bled.

The subcutaneous injections did not cause any abscess formation though there was considerable swelling, the intravenous injections caused much more trouble, and after the final dose of 10 tubes of the living culture the animal was very sick, dunging and staling repeatedly with the anus patent and exuding fluid. These symptoms, however, passed off in a couple of days.

IV. *Action of the Immune Serum.*

On comparing the action of the serum of the horse (No. 2) with that of a normal horse by mixing the respective sera with the toxin and injecting the mixture intravenously in rabbits it was at once evident that while normal serum has practically no antitoxic action the immune serum is highly antitoxic. This is shown in the following table (Table IV) which gives the results of one of a large series of tests which have been carried out with this serum. In carrying out the test 1 c.c. of a toxin, the minimal lethal dose of which for a half-grown rabbit was one-fourth of a cubic centimetre (*i.e.*, about 4 M.L.D.), was mixed with varying

quantities of the serum, the mixture made up in each case to 3 c.c. and after standing for half-an-hour in the incubator at 36° C., injected intravenously in rabbits. In the earlier experiments control tests were always made with normal horse serum but as it was found that this had no appreciable protective action these were subsequently omitted.

TABLE IV. *Testing Antitoxic Power of Serum of Horse immunised with Dysentery Toxin.*

1 c.c. Toxin = 4 M.L.D.

No. of rabbit	Weight of rabbit in grammes	Toxin	Serum	Result
1.	2080	0·15 c.c.	—	Lived, after severe paralysis
2.	1930	0·20 ,,	—	Dead (8 days), after severe paralysis and diarrhoea
3.	2010	0·25 ,,	—	Dead (2 days)
4.	2915	1·0 ,,	0·02 c.c.	Lived, no symptoms
5.	2380	1·0 ,,	0·01 ,,	,, ,,
6.	1375	1·0 ,,	0·005 ,,	,, ,,
7.	1835	1·0 ,,	0·0025 ,,	,, ,,
8.	1775	1·0 ,,	0·0014 ,,	,, ,,
9.	1245	1·0 ,,	0·0010 ,,	,, ,,

TABLE V. *Testing Antitoxic Power of Serum of Horse immunised with Toxin (Kruse).*

1 c.c. Toxin = 20 M.L.D.

No. of rabbit	Weight of rabbit in grammes	Toxin	Serum	Result
1.	560	1 c.c.	0·005 c.c.	Lived, no symptoms
2.	620	1 ,,	0·002 ,,	Lived, no symptoms
3.	630	1 ,,	0·001 ,,	Dead (2 days)
4.	690	1 ,,	0·0005 ,,	Dead (2 days)

As the limits of the protective power were not reached in the first series of experiments (Table IV) a further test was made with a much stronger toxin. The result of this is shown in Table V, where it will be seen that the serum has a very powerful antitoxic action, one five-hundredth of a cubic centimetre being sufficient to neutralise 20 minimal lethal doses of the toxin when the two are mixed and kept together for a time before injection.

The prophylactic action of the serum is particularly interesting. This is shown in Tables VI and VII. It will be seen that if the serum be given intravenously it protects absolutely against large doses of the toxin given half-an-hour later. If, however, the serum be given 24 hours before the toxin it does not protect or shows only very slight

protection. This may be due to the excretion or destruction of the antitoxin during the intervening 24 hours. If the toxin be given intravenously in one ear the animals can be saved by an injection of the serum into the other ear five minutes later.

TABLE VI. *Prophylactic Action of Immune Serum.* (*Serum given intravenously in right ear; toxin* (*K.* 8) *given intravenously in left ear half-an-hour later.*)

No. of rabbit	Weight of rabbit in grammes	Serum	Toxin (K. 8)	Result
		Immune		
1.	1345	4·0 c.c.	0·5 c.c.	Lived, no symptoms
2.	1655	4·0 ,,	1·0 ,,	,, ,,
3.	1705	4·0 ,,	2·0 ,,	,, ,,
4.	1710	4·0 ,,	2·0 ,,	,, ,,
5.	1705	4·0 ,,	2·0 ,,	,, ,,
6.	1715	4·0 ,,	4·0 ,,	,, ,,
		Control		
7.	1670	4·0 ,, normal	0·5 ,,	Dead (20 hours)

TABLE VII. *Prophylactic Action of Immune Serum.* (*Serum given intravenously in right ear; toxin* (*K.* 8) *given intravenously in left ear* 24 *hours later.*)

No. of rabbit	Weight of rabbit in grammes	Serum	Toxin	Result
		Immune		
1.	1635	4·0 c.c.	2·0 c.c.	Lived, transient paralysis in forelegs
2.	1370	4·0 ,,	2·0 ,,	Dead (4 days) after paralysis and diarrhoea
3.	1555	4·0 ,,	2·0 ,,	Lived, transient paralysis in forelegs
4.	1650	4·0 ,,	4·0 ,,	Dead (2 days)
		Controls		
5.	1970	4·0 ,, normal	0·5 ,,	Dead (40 hours)
6.	1695	4·0 ,,	0·5 ,,	Dead (20 hours)
7.	1470	4·0 ,,	0·5 ,,	Dead (20 hours)
8.	1740	4·0 ,,	1·0 ,,	Dead (40 hours)

In the earlier experiments with the antitoxic serum certain irregularities were observed in its action, a given amount of serum sometimes protecting an animal completely and at other times, though saving the animal, failing to completely avert the paralysis and diarrhoea. This was subsequently found to be due to the fact that the toxin and antitoxin require a certain time for their combination. Thus, when 3 c.c. of toxin were mixed with 0·5 c.c. of a somewhat weak serum and

the mixture injected immediately into the ear vein of a rabbit the animal died in four days after severe paralysis and diarrhoea. When the same mixture was kept in the incubator for an hour before injection the animal showed no symptoms. In order to determine the action of temperature on the time of combination two parallel series of experiments were made. In these the toxin and serum were brought to the desired temperature, mixed and kept at this temperature, for varying times, after which they were injected intravenously into rabbits. The results are shown in Tables VIII and IX.

TABLE VIII. *Showing time necessary for combination of Toxin and Antitoxin at 37° C.*

No. of rabbit	Weight of rabbit in grammes	Serum	Toxin	Serum and toxin mixed and injected after	Result
1.	1805	0·5 c.c.	3 c.c.	Immediately	Recovered after severe paralysis
2.	1725	0·5 ,,	3 ,,	5 minutes	No symptoms
3.	1525	0·5 ,,	3 ,,	15 ,,	,,
4.	1500	0·5 ,,	3 ,,	60 ,,	,,

TABLE IX. *Showing time necessary for combination of Toxin and Antitoxin at 0° C.*

No. of rabbit	Weight of rabbit in grammes	Serum	Toxin	Serum and toxin mixed and injected after	Result
1.	1550	0·5 c.c.	3·0 c.c.	4 minutes	Dead (32 hours)
2.	1480	0·5 ,,	3·0 ,,	8 ,,	Dead (42 hours)
3.	1700	0·5 ,,	3·0 ,,	15 ,,	Dead (3 days)
4.	1280	0·5 ,,	3·0 ,,	30 ,,	,,
5.	1300	0·5 ,,	3·0 ,,	60 ,,	,,
6.	1640	0·5 ,,	3·0 ,,	2 hours	Lived, no symptoms
7.	1250	0·5 ,,	3·0 ,,	5 ,,	,, ,,
8.	990	0·5 ,,	3·0 ,,	10 ,,	,, ,,
9.	1655	0·5 ,,	3·0 ,,	20 ,,	,, ,,

From these it is seen that whereas at 37° C. the combination of toxin and antitoxin is complete in less than five minutes; at 0° C. this combination requires a time of between one and two hours.

The fact that the action of dysentery toxin is not abolished by the presence of an equivalent amount of the antitoxin if the mixture is injected immediately on mixing is due to (1) the toxin and antitoxin requiring a certain time for their combination and (2) the free toxin becoming very rapidly fixed by the tissues.

Both these facts are in accordance with our experience in the case of other toxins. The credit of first drawing attention to the conditions

of neutralisation, in respect both of time and of amount of toxin and antitoxin, belongs to Fraser (1896), who investigated these points in the case of venom and antivenin. These conditions were further investigated by Martin and Cherry (1898) for venom and diphtheria toxin, by Ehrlich (1897) and by Knorr (1897) for tetanus toxin.

The rapid fixation of dysentery toxin by the tissues of the body is entirely similar to the fixation of tetanus toxin as pointed out by Dönitz (1897).

The antitoxin is very stable and is not destroyed by exposure to a temperature of 65°—66° C. for 1 hour.

From the foregoing it is evident that old cultures of the dysentery bacillus contain a soluble toxic body which is capable of giving rise in the horse to a powerful antitoxin, and the question naturally arises as to whether this toxic substance is to be regarded as a soluble toxin secreted by the bacillus into the surrounding fluid as in the case of diphtheria and tetanus or is it rather to be looked upon as more properly an intracellular toxin which has soaked out of the cell. There appears to be a large balance of evidence in support of the latter view, thus old cultures (4—6 weeks) which must contain a large number of dead and macerated bacilli are much more toxic than younger cultures (8 to 12 days) when more of the bacilli are alive. This is the reverse of what obtains in the case of diphtheria and tetanus.

Again, by autolysis of quite young cultures of dysentery highly toxic filtrates can be obtained, as was shown by Conradi (1903) and also Neisser and Shiga (1903). In order to ascertain if the toxin obtained by the autolysis of young cultures is the same as that found in solution in old alkaline broth cultures a toxin was prepared from a young agar culture by Neisser and Shiga's method. It was found that 1 c.c. of this (*i.e.*, 20 minimal lethal doses) was completely neutralised by 1 c.c. of the serum of horse No. 2, which had been immunised with the filtrates from old alkaline broth cultures, showing that the toxin is the same whether obtained from the autolysis of quite young cultures or from the growth of the bacillus for some weeks in alkaline broth. Further evidence in the same direction was obtained from the immunisation of horse No. 3, which had received only young agar cultures. The serum of this animal was tested against a toxin obtained in the usual manner by growing the bacillus in alkaline broth. The result is shown in Table X, from which it will be seen that this serum was more powerfully antitoxic than that of horse No. 2, which had received large doses of the toxin (cf. Table IV), one-thousandth of a cubic centimetre of the

serum completely neutralising twenty minimal lethal doses of the toxin.

TABLE X. *Testing Antitoxic Power of Serum of Horse immunised with Kruse's bacillus.*

1 c.c. Toxin = 20 M.L.D.

No. of rabbit	Weight of rabbit in grammes	Toxin	Serum	Result
1.	870	1·0 c.c.	0·1 c.c.	Lived, no symptoms
2.	670	1·0 ,,	0·03 ,,	,, ,,
3.	750	1·0 ,,	0·01	,, ,,
4.	650	1·0 ,,	0·003 ,,	,, ,,
5.	680	1·0 ,,	0·001 ,,	,, ,,
6.	610	1·0 ,,	0·0005 ,,	Died (2 days)
7.	570	1·0 ,,	0·0002 ,,	,,

If the dysentery bacillus is grown for 24 hours on neutral agar and the growth emulsified in saline and killed by exposure to chloroform vapour for an hour the emulsion of the dead bacilli is highly toxic for rabbits, killing them with the same symptoms as the living bacillus.

A large series of experiments was carried out in order to ascertain if the serum of horse No. 2 (immunised with the toxin only) was capable of protecting rabbits against the dead bacillus. The serum and suspension were given as follows:

(1) Serum and suspension mixed and given intravenously.

(2) Serum intravenously followed after half-an-hour by the suspension also intravenously.

(3) Serum intravenously—suspension subcutaneously at the same time.

(4) Serum intravenously—suspension intraperitoneally at the same time.

(5) Serum subcutaneously—suspension intravenously the following day.

In all the above series control experiments were made with normal serum and it was found that while the control animals all died with the typical symptoms in every case, the animals receiving the antitoxic serum were entirely protected.

V. *Comparison of the toxins obtained from bacilli of various strains and the relation of these toxins to the antitoxin.*

The formation by *B. dysenteriae* Kruse of a soluble toxin which can be neutralised by antitoxin suggests an interesting method of attacking

the question of the identity, or otherwise, of the various races of bacilli which have been described in connection with epidemics of bacterial dysentery and allied diseases. With this view nine races of bacilli were examined. The comparative agglutination of these with the serum of horse No. 3 (which had been immunised with Kruse's bacillus only) is shown in Table XI, from which it is seen that the bacilli fall into two classes:— one class, more highly agglutinated by the serum, including bacilli of Shiga, Kruse, and the three strains isolated from cases of Asylum Dysentery in England by Eyre (1904), and a second class which are less highly agglutinated, consisting of Flexner's Philippine dysentery bacillus, and two races of bacilli isolated by Duval from cases of summer diarrhoea in Baltimore and New York. The action of the bacilli on mannite (Lentz, 1902, p. 559) agrees completely with the agglutination tests, as none of the members of the first class cause any alteration in the reaction of the medium, while the four less highly agglutinated races constituting the second group all cause definite formation of acid.

TABLE XI. *Agglutination of Dysentery Bacilli with Serum of Horse No. 3 (2 hours at 36° C. and then over-night at room-temperature).*

	1/100	1/200	1/500	1/1000	1/2000	1/4000
Kruse (Král)	+ + +	+ + +	+ +	+	0	0
Shiga (Shiga)	+ + +	+ + +	+ +	+	0	0
Flexner (Dysentery, Philippines)	+	+	0	0	0	0
Flexner (Král)	+ +	+	0	0	0	0
Duval (New York, Summer Diarrhoea)	+ +	+	0	0	0	0
Duval (Baltimore, Summer Diarrhoea)	+ +	+	0	0	0	0
Eyre (No. 1, Asylum Dysentery)	+ + +	+ +	+ +	+	0	0
Eyre (No. 2, Asylum Dysentery)	+ + +	+ +	+	0	0	0
Eyre (No. 3, Asylum Dysentery)	+ + +	+ + +	+ +	+	0	0

An examination of the toxigenic power showed entirely similar results. The filtrates from one month old cultures in alkaline broth in the case of Shiga's bacillus and the three Asylum dysentery bacilli were all toxic as shown below:

TABLE XII. *Toxicity of Filtrates of Alkaline Broth Cultures (1 month old) for Rabbits (intravenous).*

	Dose	Result
B. dysenteriae Shiga	0·2 c.c.	Died (3 days)
Asylum Dysentery Eyre (No. 1)	1·0 ,,	Died (2 days)
,, ,, (No. 2)	1·0 ,,	,,
,, ,, (No. 3)	1·0 ,,	,,

all the animals showing the typical paralysis.

The effect of these toxins could, moreover, be entirely neutralised by the antitoxic serum of horse No. 2 (which had received the toxin of Kruse's bacillus only), using from four to ten minimal lethal doses of the various toxins. On the other hand the filtrates from the American bacilli in doses of 5 c.c. intravenously did not affect the animals with the exception of causing diarrhoea, which in some cases was very marked.

TABLE XIII. *Toxicity of Filtrates of Alkaline Broth Cultures (1 month old) for Rabbits (intravenous).*

	Dose	Result
B. Flexner (Philippines)	5 c.c.	Lived, diarrhoea, no paralysis
B. Flexner (Král)	5 ,,	Lived, no diarrhoea or paralysis
B. Duval (Baltimore)	5 ,,	Lived, diarrhoea, no paralysis
B. Duval (New York)	5 ,,	Lived, no diarrhoea or paralysis

These results would appear to support the views of Martini and Lentz (1902), but, on the other hand, they might be explained by the fact that in the case of the American bacilli examined the pathogenic power may have fallen to a very low value, or that, as suggested by Gay (1903), the bacilli belonging to the two types are members of a closely related group of organisms. The latter view is supported by the fact demonstrated by Gay that the antidysenteric serum is effective in different doses for both types of the microorganism.

The formation of a very definite toxin by the bacillus isolated by Eyre from a case of Asylum dysentery in England and the fact that this toxin is neutralised by the antitoxin prepared by the use of the toxin from Kruse's bacillus is particularly interesting.

The comparative action of the sera resulting from the immunisation of horses with the toxin, and with the bacillus on the infection of rabbits with the living bacillus, as well as the comparative bactericidal action of these sera *in vitro*, is at present under investigation.

The fact that the serum of animals immunised with the dysentery bacillus possesses powerful antitoxic properties appears to have been overlooked by previous workers. Shiga in 1901 published the results of his experiments with a serum obtained by immunising animals with the dysentery bacillus, and this serum was used clinically in a large number of cases with excellent results. Kruse (1903) and Gay (1902) working under Flexner also prepared a serum in the same manner and obtained good results. Both Shiga and Kruse appear to have attributed the protective action of the immune serum to its bactericidal action,

and Shiga (1903) points out that this is the first serum used for therapeutic purposes in man which fulfils the conditions laid down by Ehrlich in the Croonian Lecture of 1900, viz., that it contains an immune body for which a suitable complement exists in human serum. Shiga regards his clinical results as supporting Ehrlich's view, but the question of the serum being antitoxic, as well as bactericidal, does not appear to have been considered.

Gay (1902) showed that his serum protected against single lethal doses of vaccine, but though in his second paper (1903) he says that bacteriolysis is not an index of the protective power *in vivo* of antidysenteric serum he makes no statement as to the important antitoxic properties possessed by this serum[1].

CONCLUSIONS.

1. Old cultures of *B. dysenteriae* Kruse in somewhat highly alkaline broth contain a soluble toxin.

2. The same toxin is also contained in the bodies of the young bacilli.

3. The horse and rabbit are very highly susceptible to this toxin, the guinea-pig, rat, and mouse being very resistant.

4. Immunisation of the horse, either with the soluble toxin from old alkaline broth cultures, or with the bodies of the young bacillus, gives rise to an antitoxin.

5. The antitoxic power of the serum of horses so immunised may reach a very high value—in the case of an animal immunised with the bacillus one-thousandth of a cubic centimetre of the serum being sufficient to protect a small rabbit against twenty minimal lethal doses of the toxin.

6. The antitoxin is capable of protecting animals, either when mixed with the toxin, or when given separately at another part of the body either at the same time or shortly before or after the toxin.

7. The toxin and antitoxin require a certain time for their combination *in vitro*, and this time is dependent upon the temperature,

[1] *Note during preparation.* In a paper published quite recently Rosenthal (1904) describes a serum which he has obtained by injecting horses with both the toxin and cultures; this serum possesses both antitoxic and bactericidal properties, and he has been able to use it in the treatment of 157 cases of bacterial dysentery with most encouraging results.

varying from less than 5 minutes at 37° C., to between one and two hours at 0° C.

8. Shiga's Dysentery bacillus and three strains of a bacillus isolated from cases of Asylum Dysentery in England by Eyre were found to yield a similar toxin, and this toxin was neutralised by the antitoxin prepared by means of the toxin from Kruse's bacillus. A strong point in favour of the identity of the above bacilli.

9. Attempts to obtain a soluble toxin from *B. dysenteriae* Flexner (adult dysentery, Philippines) and from two races of the bacillus isolated from cases of summer diarrhoea by Duval in Baltimore and New York were unsuccessful.

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ON THE MECHANISM OF AGGLUTINATION.

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In view of the large number of hypotheses advanced to account for the phenomenon of agglutination of bacterial cells, it was suggested to me by Dr Allan Macfadyen, that I should investigate the agglutination of typhoid bacilli from a physico-chemical standpoint. I here beg to express my indebtedness to Dr Allan Macfadyen and Dr George Dean for much kindly aid and criticism.

In a preliminary notice to the Pathological Society (13. II. 1904), I indicated that my results supported the view first advanced by Bordet, that the phenomenon of agglutination may be divided into two stages:

1. Fixation of agglutinin by bacilli.
2. Aggregating influence of salts and other substances.

Further, I suggested that the fixation obeyed the general laws of staining and that the aggregation was a surface-tension effect. In this investigation the first question was whether agglutination was a strictly specific phenomenon or one of a general character. Agglutination is not entirely specific. According to Köhler (1900) typhoid bacilli are agglutinated not only by typhoid immune serum but also to a smaller extent by the sera obtained from *B. coli*, pneumococcus and meningococcus infections. Lubowski and Steinberg (1904) obtained agglutination by immune sera from proteus bacilli and staphylococcus, and Rodet (1904) found that the normal serum of the rabbit, etc. if not too much diluted agglutinated typhoid bacilli. Stern (1903) has shown that the serum produced by a bacillus generally agglutinates allied bacilli but is specific in the sense that the bacillus itself is most easily agglutinated. In addition to the specific agglutination caused by immune sera, a non-specific agglutination can, according to Malvoz (1897), be brought about by saffranin and other dyes, and Gilardoni (1903) states that, in general, any reagent which precipitates proteids will cause agglutination.

Since the most diverse fluids thus lead to the same end the reaction cannot be regarded as a definite chemical change, and further investigation is required to ascertain whether the physical part of the reaction (or mechanism) is the same in all cases.

The chief views regarding the essential nature of agglutination which have been put forward are those of Pfeiffer, Gruber, Paltauf, and Bordet.

(1) Pfeiffer (1896) and Emmerich and Löw (1899) regarded agglutination as a vital paralysis of the bacilli due to the action of a bacteriolytic enzyme. It has, however, been shown by Joos (1901) that the agglutinating substance in typhoid immune serum is in the absence of salts absorbed without diminishing the motility of the bacilli, and further, that after the agglutination is completed by the addition of an electrolyte, *e.g.* sodium chloride, reproduction does not cease. Moreover, the enzymic character of the agglutinating substance, or agglutinin, is rendered doubtful by the fact that the bacteriolytic enzymes are destroyed by temperatures at which the agglutinins remain unaffected.

(2) Gruber (1899), Dineur (1898), and Nicolle (1898), supposed that a glutinous substance, "glabrificin," was absorbed from the serum by the bacilli, thereby causing small adhesive prominences on the membranes, or rendering the flagella, or the membranes themselves, adhesive on contact.

These hypotheses, which ascribe agglutination to the adherence of bacilli after contact, are insufficient, however, to account for the movement of dispersed non-motile bacilli towards a common centre.

(3) Paltauf (1897) and Duclaux considered that a specific precipitate is produced in the medium which during flocculation mechanically carries the bacilli with it.

Wassermann (1903) and Kirstein (1904) have shown that the precipitable substance in a culture filtrate and the agglutinable substance in the bacilli are very closely related, and that the former may be regarded as agglutinable substance ejected by the bacilli into the medium.

Beljaeff (1904), however, finds that the agglutinating power of a typhoid immune serum is not parallel to its power of precipitating a typhoid filtrate, and Norris (30. v. 1904) considers that many facts point to at least some of the substances concerned in specific agglutination and specific precipitation being distinct.

That agglutination can be brought about by a visible precipitate

is certain, and that extremely fine precipitates are sometimes formed round the isolated organisms during agglutination, has been shown by Löwit (1904), but agglutination of *B. typhosus* has been observed by Hinterberger where no precipitate was demonstrable, and it remains an open question whether precipitation in the medium is a necessary part of the mechanism of agglutination. As regards precipitation within the bacilli it will be seen from the following (part 2) that this is highly probable.

(4) Bordet (1899) separated the mechanism of agglutination into two stages as mentioned above, (1) fixation, and (2) aggregation. The fixation of agglutinin by the bacilli he considers to be analogous to the fixation of a dye by a tissue, and that once the agglutinin is fixed, the bacilli obey the laws of inert particles, and that aggregation is caused by changes in the molecular attraction between the organisms and the surrounding medium.

Since staining is usually regarded as a special case of adsorption, *i.e.* the condensation or concentration of gases or dissolved matter on substances with highly developed surfaces, one might expect that the fixation of agglutinin would obey the general laws of adsorption. If the second part of the hypothesis is correct, since the relations between inert particles and a surrounding medium are directly governed by the surface tension of the particles against the medium, one would expect the bacilli to be subject to corresponding surface tension effects.

It being, therefore, possible to control both parts of Bordet's view by direct experiments on adsorption and surface tension, an investigation on these lines was instituted.

The Fixation of Agglutinin.

According to Arrhenius (1903) agglutinin is partitioned between bacilli and medium much as iodine is between water and carbon-disulphide. He further deduces that the molecular weight of agglutinin dissolved in the medium is one and a half times that of the agglutinin dissolved in the bacilli.

The following objections may be raised to the views advanced by Arrhenius. Specific agglutinins and the agglutinable substances, precipitins and precipitable substances, toxins and antitoxins, belong to the same class of substances. (Bordet, 1903.) This class also includes lysins and antilysins, immune bodies and complements with their antibodies. All these substances belong to the division of

colloidal solutions of compounds of high molecular weight. (Müller, 1903.)

Toxins, lysins, and agglutinins are possessed of some degree of diffusibility, but in general the substances with which they react have a very low rate of diffusion of the order of magnitude of that of globulin and other typical colloids. (Billitzer, 1903.) A recent determination of the molecular weight of a well-purified typical organic colloid, viz., Glycogen, by the Nernst-Abegg cryoscopic method according to Gatin-Gruzewska (1904), gave no depression of the freezing-point of the aqueous medium. Glycogen is then either a difficultly soluble substance with a molecular weight over 140,000, or it is incapable of forming a true solution and its molecular weight *indefinite*. This glycogen migrated in an electric potential gradient like other colloids, to the anode. Sabanejev had previously found for glycogen the molecular weight 1620, probably owing to the presence of crystalloidal impurities.

E. W. Reid (1904) using Starling's (1899) method found that by washing salted out or crystallised proteids, solutions of proteids are obtained which give no osmotic pressure against a membrane of gelatine.

The view that colloidal solutions of high molecular organic compounds are not in homogeneous solution is supported by their dispersion of polarised light. Raehlmann (1904), Römer and Siebert (1904), using the Siedentopf-Zsigmondsky microscope, have observed in serum and other albumin, globulin, and glycogen solutions a similar granular appearance to that exhibited by colloidal gold, silver, and platinum. Taken in conjunction with the low diffusibility and absence of osmotic pressure this granular structure seems to indicate that typical colloidal proteids are almost entirely in suspension, and that the pseudo-solutions are really heterogeneous systems. The conditions under which the laws of Boyle, Gay-Lussac, and Avogadro apply being absent, it is probable that for proteids of this class the application of the gas laws in the manner made by Arrhenius is not a true interpretation of the mechanism of their reactions and equilibria with other substances. Nernst (1904) has recently pointed out that for reactions in heterogeneous systems, in so far as they take place entirely or partly on the interfaces of the different phases, the velocity of reaction depends entirely or partly on velocities of diffusion, which in general have nothing to do with the order of a reaction, that is, with the number of reacting molecules. Since at least one of the substances in the reactions with which we are concerned must be considered as in the typical colloidal state

van't Hoff's laws cannot be applied to the reactions of specific substances with their antibodies.

On the other hand, typical colloidal solutions being heterogeneous systems with highly developed interfaces, the velocities of their reactions with other substances, and the equilibria they attain, should be governed by the laws which hold for the absorption of substances from solutions by suspensions, porous substances, and highly developed surfaces in general, classed generally as adsorption phenomena.

That there are quantitative laws of adsorption has been fully recognized by Ostwald (1891). A dilute solution of hydrochloric acid digested with animal charcoal slowly reaches equilibrium, which does not change when portions of the solution or of the charcoal are removed. The equilibrium is then only dependent upon the ratio of the concentration of hydrochloric acid in the solution to that in the charcoal. On the addition of water the charcoal gives up a portion of its hydrochloric acid within a few minutes and a new equilibrium is established. A given mass of charcoal can be obtained in this manner so uniform in concentration as regards hydrochloric acid that the experiments agree within fractions of a percentage. With various substances, the amounts absorbed by charcoal, when plotted as ordinates against the amounts of water as abscissae in right-angled coordinates, gave hyperbolic curves. The laws governing the absorption of the most varied substances are the same, yet, in many cases the charcoal undoubtedly reacts chemically on the substance absorbed. It is important to observe that the state of equilibrium is easily and rapidly attained when the solid body is treated with a concentrated solution of the substance to be absorbed and subsequently by addition of water brought to the desired dilution. On the contrary, if the solid substance be brought into contact with the already diluted solution days may elapse before equilibrium is attained.

A further important feature of adsorption equilibria is that the general formula

$$C_1 = KC_2^n$$

is capable of wide application, where C_1 is the concentration of the free substance in the aqueous medium, C_2 the concentration of the bound substance in the adsorbing material, K and n constants dependent on the chemical nature of the substances brought into contact. Thus when a solution of iodine is mixed with animal charcoal the relation is

$$C_1 = 0{\cdot}0014\, C_2^4.$$

Schmidt (1904) found that acetic, succinic, and oxalic acids with charcoal, similarly give definite and characteristic constants K and n. It may be well to observe that the values of n and K do not give any definite idea of the magnitude of the adsorbed molecule or grain as compared with that in the aqueous medium but rather of the relative intensity and capacity of adsorption of charcoal and water for the substances partitioned.

It has been observed that colloids are adsorbed by charcoal, which leads to the consideration of the more immediately interesting case in which an inferior colloid or a crystalloid is adsorbed by a typical colloid.

For this we can assume within certain limiting concentrations that

$$C_1 = KC_2^n$$

where C_1 is the concentration of inferior colloid that is retained in the aqueous medium as a consequence of the solvent-like attractive or adhesive intensity of the latter, C_2 is the concentration of inferior colloid that is bound or taken up by the typical colloidal medium in virtue of its adhesive intensity.

From Eisenberg and Volk's (1902) experimental investigation of the equilibria which obtain between agglutinin and typhoid bacilli, Arrhenius has deduced the relation

$$\frac{C}{A} = KB^{\frac{2}{3}}$$

where $\frac{C}{A}$ represents the concentration of agglutinin in the bacilli, B the concentration of free agglutinin in the medium, and K is a constant. This equation is, it seems to me, a special case of the adsorption formula where the power $\frac{2}{3}$ has only significance in indicating the relative intensity of adsorptive power of the medium and the bacilli for agglutinin. The validity of the special formula given is however questionable, for in calculating the agglutinating value of a serum an assumption has been made by Eisenberg and Volk which is very open to criticism. They take it for granted that a serum which gives a concentration of agglutinin per c.c. equal to one 24 hours' unit when diluted to 20,000 times its original volume, will contain in 1 c.c. of the original serum 20,000 units of agglutinin. In other words, they assume that the number of agglutinin units present in a fluid is independent of its volume, no association, dissociation of the units, or, in other words, no change in the binding capacity of the agglutinin units takes place. When we consider that flocculation by sera and other chemicals reaches

a maximum at certain concentrations, *i.e.* that there are optimum concentrations of sera, etc., above and below which the flocculation is diminished, it seems to me, that without further experimental investigation, we cannot assume that the fixation of agglutinin is unaffected by this phenomenon or that the binding capacity of the diluted unit is equal to that of the same quantity of agglutinin when in the concentrated state. The experimental error being the same for the estimation of free agglutinin in dilute and concentrated solutions, the deviations from the calculated values for dilute solutions shown by Eisenberg and Volk's figures prove that Arrhenius's formula, as it stands, does not embrace the entire range of dilutions. In Table III (Arrhenius, 1903, p. 417), assuming that in more dilute solutions we have a different relation, we obtain a better constant by using the formula

$$\frac{C}{A} = KB^{\frac{3}{4}} \text{ for the more concentrated solutions, viz.,}$$

$$K = 13{\cdot}6,\ 12{\cdot}3,\ 12{\cdot}5,\ 12{\cdot}54,$$

whereas

$$\frac{C}{A} = KB^{\frac{2}{3}} \text{ gives } K = 23{\cdot}2,\ 24{\cdot}8,\ 26{\cdot}3,\ 28{\cdot}2.$$

Again, rejecting according to the principle adopted by Arrhenius the last value of Table XIII we obtain for $\frac{C}{A} = KB^{\frac{1}{2}}$, $K = 23, 17, 23{\cdot}2, 24{\cdot}5$, which seem to be as good as $\frac{C}{A} = KB^{\frac{2}{3}}$, $K = 12{\cdot}7,\ 11{\cdot}8,\ 8{\cdot}4,\ 10{\cdot}6$. But a change in the power to which B is raised would give from Arrhenius's standpoint a radical change in the molecular relations of agglutinin, for then the molecular weight of free agglutinin would be to that of bound agglutinin as 4 is to 3 or 2 to 1, entirely different dissociations to that found in other cases. From the adsorption point of view the variation of K and n from one serum to another is to be expected. From the hypothetical gas-law standpoint, however, K and above all n must be constant. Moreover, as Arrhenius himself points out, if multiples of a certain concentration of agglutinable substance and agglutinin be brought together the power $\frac{2}{3}$ is incompatible with Eisenberg and Volk's experimental series in which they find the ratio between agglutinin bound and free to be unchanged on bringing multiple concentrations together. It seems to me that this last result of Eisenberg and Volk would be explicable on the partition formula $\frac{C}{A} = KB$, and this would lead to the conclusion that n was dependent

upon the conditions of the experiment. The apparently constant active mass of the agglutinable substance within the bacilli cannot be ascribed to the non-participation of the cell contents in the reaction, for, as we have seen, Wassermann (1903) has demonstrated that agglutinin reacts with precipitable substance and gives rise to a precipitum, and we have no reason to assume that this reaction does not occur within the bacilli when agglutinin is absorbed; on the contrary, the second phase of agglutination is most easily accounted for by the assumption that the cell contents experience a decided change in structure, either physical or chemical, on the absorption of agglutinin. From a physico-chemical point of view it does not then seem probable theoretically that the gas laws apply to the fixation of agglutinin by agglutinable substance, nor experimentally that there is foundation for the application. As far as the equilibria are concerned, a superficial analogy is, however, to be expected, such as the phenomena of adsorption would lead us to expect. With respect to the more general problem of the velocity of reaction of substances which may be classed with agglutinin, and agglutinable substance such as toxin and antitoxin, the agreement between the experimentally observed relations and those calculated by Arrhenius is also merely superficial. Similar agreement between observations on the velocity of reaction in heterogeneous systems and calculations on the assumption of homogeneity has been observed by Hantsch (1904), who recognised fully the untenability of the assumption, for the absorption of ammonia gas by solid organic acids and of hydrogen chloride by solid amine bases.

With a view to the elucidation of the class of phenomena to which the fixation of agglutinin may be attributed the following experiments were carried out:

The unit concentration of agglutinin was arbitrarily fixed as that dilution of a serum 1 c.c. of which on addition to 1 c.c. of a standard suspension of typhoid bacilli in a tube 6 mm. in diameter showed clumps just visible with the aid of a hand-lens at the end of $2\frac{1}{2}$ hours, at room temperature. The same lens was used throughout the series compared. In thus reducing the time at which the reading took place I hoped to eliminate part of the error due to the growth of a living culture in a very weakly agglutinating fluid. In Eisenberg and Volk's experiments this error may have influenced their results, for they allowed their test mixtures to remain 24 hours before reading. The absolute quantity of agglutinin represented by a unit depends upon the time given to the test mixture to flocculate, a $2\frac{1}{2}$ hours' unit contains more agglutinin

than a 24 hours' unit, hence when an agglutinating value is ascribed to a fluid the time value of the unit must be given.

The standard suspension was prepared from an agar culture of a good agglutinating strain, grown at 37° C. for 18 hours. Each agar tube was rinsed out with 15 c.c. of 0·82 % sodium chloride. To secure uniformity the suspensions, obtained from a dozen tubes, were mixed and filtered through glass-wool to remove agar particles.

The typhoid immune serum first employed was obtained from Horse No. 1 on December 5th, 1903, and gave agglutination at a dilution of 1 in 40,000, equivalent to an agglutinating value of 40,000 diluted 2½ hour units.

Since the use of killed cultures might reduce the experimental error, a few comparative series, to test the relative agglutinability of the above suspension and of the standard suspension after it had been heated to 58° C., for 30 minutes, were carried out and gave as a mean the following result. With the unheated standard suspension :—

4 Series.

Serum concentration	1/20	1/200	1/2,000	1/20,000	1/40,000
Time of agglutination	8	19	29	40	150 minutes

With the heated standard suspension :—

4 Series.

Serum concentration	1/2,000	1/8,000	1/20,000
Time of agglutination	10	80	150 minutes

The serum in the higher dilutions, corresponding to the 2½ hour units, showing from this only half the agglutinating power on the heated bacilli of that which it has for the unheated, I decided to use the unheated bacilli, as they form a more delicate indicator of diluted free agglutinin in a solution. Again, since heating probably alters the nature of the absorbing substance and since from the above it seems probable that the laws which hold for the fixation of agglutinin by the heated agglutinable substance would be different to those governing the fixation in the unheated substance, the unheated standard suspension was also used for the experiments on equilibrium. A further investigation to ascertain whether the motility of the bacilli was connected with their agglutinability gave entirely negative results. Two non-motile cultures obtained by 18 transfers on agar at 42° C., gave values for their agglutinability which were, within the experimental error, identical with those given above for the unheated standard suspension. On staining

by Loeffler's method the flagella were observed in this non-motile culture to be still attached to the cell bodies. That the contents of the cell play an important, if not the chief, part in the fixation of the agglutinin seems to be indicated by the fact that one sample of typhoid residue obtained by grinding the organisms at the temperature of liquid air and washing repeatedly with distilled water only gave agglutination with the above immune serum in the concentration of 1/20; a second sample did not agglutinate even with the undiluted serum. This result is in entire harmony with Bordet's view.

With regard to the minutiae of the experimental method it is important that after mixing bacilli and agglutinating fluid the mixture be not disturbed. To trace the possible effect of shaking, the heated culture, which with serum in a concentration of 1 in 2,000 agglutinated in 10 minutes, was shaken up for 1 minute, the bacilli reagglutinated in 1 hour; on repeating the agitation a third agglutination took place in 3 hours. This result is also interesting in showing that the second phase of agglutination, *i.e.*, the aggregation, is quite distinct from the fixation of agglutinin which is completed in a very short time.

The equilibria existing between various concentrations of serum and a constant concentration of bacilli were then determined:—10 c.c. of various dilutions of serum were added to equal volumes of suspension, thoroughly mixed and allowed to stand 3 hours at 17° C., the whole was then centrifugalised, the supernatant fluid decanted and the dilution in which it just caused agglutination in $2\frac{1}{2}$ hours determined. The results obtained expressed in the manner adopted by Eisenberg and Volk and the value of K calculated by Arrhenius's formula were as follows:—

Serum of Horse No. 1 = 40,000 $2\frac{1}{2}$ *hour units.*

Mean values obtained from 6 Series.

Concentration of serum	Units of agglutinin added	Units of agglutinin absorbed	Units of agglutinin free	K
1/20	2,000	1,300	700	16·5
1/30	1,333	1,133	200	38·9
1/40	1,000	840	160	28·5
1/50	800	768	32	76·2
1/60	666	646	20	87·6
1/200	200	200	0	—

With the exception of the 1/30 dilution the value of K calculated according to the formula of Arrhenius shows a constant increase.

Taking Eisenberg and Volk's (1902) agglutinating unit, viz.—the dilution of agglutinating fluid 1 c.c. of which on addition to 1 c.c. of

the above-mentioned bacterial suspension showed a definite sediment after 24 hours—and using another serum, less regular results were obtained. 15 c.c. of suspensions were mixed with 15 c.c. of serum,

$$S, \frac{S}{2}, \frac{S}{4}, \frac{S}{8}, \frac{S}{16}, \frac{S}{32}, \frac{S}{64}, \frac{S}{128}, \frac{S}{256}, \text{ and } \frac{S}{512}.$$

The highest concentration of serum was obtained by adding 30 c.c. serum to one agar tube of culture. The values obtained were as follows:—

Serum of Horse "Tom" = 44,000 24 hour units.

Mean values obtained from 4 Series.

Concentration of serum	Units of agglutinin added	Units of agglutinin bound	Units of agglutinin free
1/1	44,000	24,000	20,000
1/2	22,000	8,000	14,000
1/4	11,000	3,000	8,000
1/8	5,500	500	5,000
1/16	2,750	750	2,000
1/32	1,375	675	700
1/64	688	488	200
1/128	344	329	15
1/256	172	170	2
1/512	86	86	0
1/1,024	43	43	0

The mixtures were heated for 2 hours at 37° C., and then allowed to stand 24 hours. No constant could be found by Arrhenius's formula for these figures.

Owing to the wide deviations obtained from Arrhenius's formula the question arose whether equilibrium between bound and free agglutinin could be easily and certainly attained or whether we had here only an apparent or so-called "false" equilibrium. The serum of the horse "Tom" (S) was brought together with a suspension of typhoid bacilli (T) of a concentration equal to one agar tube of culture grown for 18 hours at 37° C., in 5 c.c. of saline. The concentrations $\frac{S}{3}$ and $\frac{T}{3}$ were obtained by a dilution with saline. The final volume was 30 c.c., and the final concentration of serum and bacilli in all cases was the same. The following mixtures were made:—

2 Series.

I. 15 c.c. $\frac{S}{3}$+15 c.c. $\frac{T}{3}$.

II. 15 c.c. $\frac{S}{3}$+ 5 c.c. $\frac{T}{3}$, after 18 hours+10 c.c. $\frac{T}{3}$.

III. 15 c.c. $\frac{S}{3}$+ 5 c.c. T, after 18 hours+10 c.c. saline.

IV. 15 c.c. $\frac{T}{3}$+ 5 c.c. $\frac{S}{3}$, after 18 hours+10 c.c. $\frac{S}{3}$.

V. 15 c.c. $\frac{T}{3}$+ 5 c.c. S, after 18 hours+10 c.c. saline.

VI. 5 c.c. T+ 5 c.c. S, after 18 hours+20 c.c. saline.

VII. 5 c.c. T+ 5 c.c. S, after 2 hours+20 c.c. saline.

VIII. 15 c.c. $\frac{T}{3}$+15 c.c. $\frac{S}{3}$, mixed at the time of addition of the second portion in the other cases.

After allowing to stand for 24 hours further, the supernatant fluids had practically the same agglutinating values, the variations being at the most 200 units in 8,000 free units of agglutinin measured by bringing 1 c.c. of the fluid together with 1 c.c. of $\frac{T}{3}$ and heating to 37° C., for 2 hours.

Given a considerable time then we attain a fairly constant equilibrium with a concentrated serum. This equilibrium was found, however, to show the characteristics of "false" equilibria, when the serum was more diluted. *The successive addition of the bacillary suspension to the serum removed more agglutinin from the solution than when the addition was made at one time.* 10 c.c. of the diluted serum of Horse No. 1 were added to 10 c.c. of the standard suspension at once, and in a second case at the rate of 5 drops every 5 minutes. The concentrations of serum used were $\frac{S}{10}$ and $\frac{S}{200}$. The equilibria seemed to be, within the experimental error, uninfluenced by the rate of addition. When, however, 10 c.c. of the suspension were gradually added to an equal volume of the $\frac{S}{10}$ concentration of serum, the free agglutinin corresponded to 95 units ($2\frac{1}{2}$ hr.) when the addition was made at one time, whereas for the addition in parts 60 units ($2\frac{1}{2}$ hr.) were left free.

The serum of the horse "Tom" of concentration $\frac{S}{10}$ was then mixed with the standard suspension in the following manner:—

2 Series.

I. 15 c.c. $\frac{S}{10}$+15 c.c. suspension, centrifugalised after 3 hours.

II. 15 c.c. $\frac{S}{10}$+gradually increasing fractions of suspension.

III. 15 c.c. suspension+gradually increasing fractions of $\frac{S}{10}$.

IV. 15 c.c. $\frac{S}{10}$+15 c.c. suspension, centrifugalised after 30 minutes.

In II and III the entire addition of the second 15 c.c. was spread over $2\frac{1}{2}$ hours and the mixtures centrifugalised after a further 30 minutes. The supernatant fluids of I, III and IV showed 2,600 units of free agglutinin in a 2 hours' test at 37° C., whereas II showed 2,400 units; I, III and IV gave 3,200 six-hour units and II 2,800. Since I and IV gave the same result the agglutinin equilibrium is set up in less than 30 minutes at room temperature. No. II confirms the view that we are dealing with a "false" equilibrium.

It cannot then be assumed that the gas laws apply to the partition of agglutinin between bacilli and medium, nor that the formula $\frac{C}{A}=KB^{\frac{2}{3}}$ expresses the relation, and in the application of the general formula $\frac{C}{A}=KB^n$ K and n will probably be dependent upon the conditions of the experiment, such as the manner in which serum and bacilli are brought together, the temperature, the strain of bacilli used, their age, and the constituents of the serum other than agglutinin.

Since these results agreed well with the conception of the fixation of agglutinin advanced by Bordet, I carried out a staining experiment which lends further probability to the view that the mechanisms of both have much in common. In these experiments the staining substance (fuchsin) corresponds to the agglutinin, and the bacillary substance is represented by absorbent paper.

A few drops of saturated alcoholic fuchsin were added to a litre of water. 100 c.c. of water and 100 c.c. of this solution were placed in a porcelain tray A, 100 c.c. of water and 33·3 c.c. of the solution in a tray B. A piece of thick absorbent paper of superficial area nearly equal to that of the bottom of the tray was placed in A and a similar

piece in B; the paper weighed 4·4 grammes in each case. After an interval of 24 hours a similar experiment A^1 was started, and at the same time 66·7 c.c. of fuchsin solution were added to B. The three systems were then allowed to stand a further two hours and the intensity of colour of the supernatant fluids compared. A was slightly less coloured than A^1, but B was strongly coloured. On diluting 5 c.c. of B until it had the same intensity as A^1 7·5 c.c. of water had to be added. B, therefore, contained 2·5 times the concentration of free fuchsin in A^1.

Similarly the addition of the absorbent paper in parts removed more fuchsin from the solution than in the case where the whole amount of paper was added at once.

Von Dungern (1904), and Sachs (1904), have found similar false equilibria to exist in the relations between toxin and antitoxin. Indirectly then we have further support for the view that the gas laws are not applicable to specific substances and their antibodies, and the view that we are here dealing with adsorption phenomena appears to be confirmed.

Summary.

1. The hypotheses of Pfeiffer, Emmerich and Löw, attributing agglutination to a vital paralysis due to the action of a bacteriolytic enzyme, and those of Gruber, Dineur, and Nicolle, which ascribe the action to the glutinous nature of the membranes or cilia, are insufficient to account for the observed phenomena.

2. The views of Paltauf and Duclaux that a specific precipitate is formed in the medium which mechanically carries the bacilli together are sufficient, but probably do not account fully for the agglutination of washed bacilli.

3. Arrhenius's assumption that the gas laws are applicable to the partition of agglutinin between bacilli and medium is improbable since the conditions under which these laws can be applied are absent.

4. The formula given by Arrhenius for the partition of agglutinin seems to be a special case of a general formula holding for the absorption of substances from solution by substances with highly developed surfaces, *e.g.*, the adsorption of iodine from solution by charcoal. A superficial analogy between the gas partition law and the adsorption partition law for equilibria is to be expected, likewise an equally superficial analogy between reactions in true solutions and colloidal solutions, *e.g.*, of agglutinin, for the velocity of reaction.

5. The special formula given by Arrhenius does not apply to the entire range of agglutinin solutions and the change which must be made in the constants is incompatible with the application of the gas laws, but agrees with the view that the fixation of agglutinin is due to adsorption.

6. The fixation of agglutinin from two different typhoid immune sera by living typhoid bacilli did not correspond to the partition law deduced by Arrhenius from the experiments of Eisenberg and Volk.

7. The rate of addition of bacillary suspension to agglutinating serum is a factor determining the amount of agglutinin fixed by the bacilli. By adding the suspension in parts more agglutinin is removed than in the case where the whole amount of suspension is added at once. This points to the equilibria belonging to the class met with in absorption.

8. Similar adsorption equilibria are obtained by experiments on staining.

9. The cell contents probably play an important part in agglutination as the washed membranes are but slightly agglutinated.

10. Motile cultures of *B. typhosus* grown at 37° C., and non-motile cultures grown at 42° C., agglutinated equally well, the agglutinable substance probably being unchanged.

11. The law governing the fixation in *B. typhosus*, heated to 58° C. for 30 minutes, is probably different to that holding for living cultures owing to modification of the agglutinable substance.

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THE PRESENCE OF BACTERIA IN THE ORGANS OF HEALTHY ANIMALS.

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The possible occurrence of bacteria in the organs of normal animals has been made on many occasions a subject of inquiry. The question is obviously an important one whether considered clinically or scientifically. The interior of the healthy organism has usually been regarded as free from bacteria and the researches of Pasteur and of Duclaux have supported this conclusion. A number of observers have brought forward additional and confirmatory evidence. On the other hand, a recent research published by Ford[1] furnishes, according to this author, experimental proof that normal organs do, as a matter of fact, contain bacteria. The paper gives a valuable *résumé* of the literature on the subject and from this I will quote before dealing with the author's own experiments. Meissner found no bacteria capable of development in the tissues of the healthy animal, whilst Zweifel concluded that germs were habitually present but of an anaerobic character and limited in their capacity for development by the amount of oxygen in the blood stream. The researches of Hauser led to a negative result, the microscopic examination and the cultures made from healthy tissues showing them to be free from bacteria in every case. Welch only found the colon bacillus in the internal organs in cases of distinct lesion of the intestinal mucosa and he further states that this bacillus does not invade the blood and

organs during post-mortem decomposition. Neisser, employing the usual precautions to avoid contamination, removed the liver, spleen, kidneys, heart, lungs, and mesentery from rabbits and guinea-pigs and placed the tissues in nutritive media. The subcultures made from these at the end of two days were invariably found to be sterile. Neisser concludes "that under normal conditions no bacteria are present in the lymph or blood stream." He found, on the other hand, that after producing artificial lesions of the intestines and feeding the animals experimented on with cultures of various micro-organisms the organs of such animals contained either these bacteria or representatives of the intestinal flora. Opitz carried out experiments on cattle in the abbatoir at Breslau. Portions of the mesenteric glands from cattle were carefully sterilised on the surface and small pieces were then excised and cultivated upon agar and gelatin plates. It was found that at the end of three days no bacteria had developed in the majority of the tissues tested whilst those which were isolated from a small number of decomposed tissues were spore-bearing organisms—e.g., bacillus subtilis. Opitz concluded that the mesenteric glands of cattle are normally sterile and that bacteria do not pass through the healthy intestinal wall during digestion. Ford emphasises the fact that in his personal experiments the incubation of the tissues removed by him was prolonged for 17 days and that organisms were found to grow in the cultures as late as the seventeenth day. Neisser and Opitz, on the other hand, drew their conclusions from specimens which had only been incubated for a period of three days. Ford in a series of experiments made with 30 organs removed from rabbits found that 66 per cent. contained bacteria cultivable on agar, gelatin, and broth, whilst with 18 organs removed from guinea-pigs positive results were obtained in 61 per cent. The bacteria found in each case were identical and appeared in the culture media on the seventh day. It was thus only after a lapse of seven days

that the organisms detected by Ford escaped from the organs which contained them and appeared as a growth in the culture soil. The bacteria found were staphylococcus albus and aureus, bacillus mesentericus, bacillus proteus, bacillus subtilis, and bacillis colli communis.

The organs of cats showed a bacterial flora different from that of the rabbits and guinea-pigs. Of 18 organs from the cat positive results were obtained in about 77 per cent. Staphylococci and colon bacilli were detected but not the bacillus subtilis or the bacillus mesentericus. The bacillus proteus, bacillus megatherium, and bacillus mycoides were found as well as two spore-bearing bacilli detected in the broth cultures. These spore-bearing forms along with the bacillus megatherium and bacillus mycoides and at times bacillus Zopfii were also present in the organs of dogs. The experiments of Ford showed that out of 96 organs taken from 34 animals 69 per cent. contained bacteria and that less than 30 per cent. were sterile. It appeared advisable in view of the exceptional character of these results to repeat the experiments and I will now briefly describe the methods which I adopted and the results. Every precaution was taken in the course of the experiments to avoid aerial or other accidental contamination. A rabbit was killed, the skin was deflected from a central linear incision, and the abdominal muscles were seared with a red-hot iron. The abdomen was opened with a sterilised scalpel and ten cubic centimetres of peptone broth were poured over the intestines and immediately drawn up, together with any peritoneal fluid, by means of a sterile syringe. The fluid obtained in this way was placed in a sterile tube fôr subsequent cultivation. The intestines were next drawn aside by sterile glass rods and the exposed organs or portions were removed—viz., the kidney, the suprarenals, the spleen, the liver, and a few of the mesenteric glands. Each organ was quickly passed through the flame of a Bunsen burner and then divided, one portion being dropped

into a flask of nutrient broth and the other into melted agar cooled down to 45°C. The organs in the broth were broken up with a sterilised knife and both broth and agar were incubated at blood heat. The broth cultures were employed to test for aerobic and the agar cultures for the growth of anaerobic micro-organisms. After 17 days' incubation portions of the organs planted in the agar were removed with a platinum needle, transferred to broth tubes, and kept for several days at blood heat under anaerobic conditions in a modified Buchner's tube and eventually examined for bacterial growth. From the organs in broth, subcultures were made in fresh broth every third day, care being taken to avoid contamination. These subcultures were kept at blood heat, and in the event of no growth occurring were re-inoculated on the second or third day. 80 organs and the peritoneal fluid from ten rabbits were tested in the above manner. Growths were obtained from 23 organs in the aerobic broth cultures and from one rabbit two organs gave a growth of the colon bacillus. This latter result was, however, probably due to an accidental injury to the intestine and a consequent contamination of the surface of the organs. In no case was a growth of the colon bacillus obtained from an animal with uninjured intestines. The bacteria present in the broth cultures were non-pathogenic forms and their intraperitoneal injection in rabbits proved harmless. They consisted mostly of such common types as the bacillus mesentericus vulgatus along with a number of other non-pathogenic spore-bearing forms. These organisms never appeared later than the third day in the cultures and differed in no respect from similar organisms planted *per se* in peptone broth. From the anaerobic agar cultures no growth was obtained in any instance and I was unable to confirm Zweifel's statement as to the presence of anaerobic organisms in the healthy body tissues. Similar experiments were made with seven organs from the cat and a similar

number from the dog. Aerobic growths of non-pathogenic forms were obtained in three out of seven organs in each case.

On comparing these results with those of Ford, one is struck with the similarity of the organisms found and with the fact that the percentage is much lower than that obtained by him. The number of organs tested by myself was 80 and excluding the two cultures of the colon bacillus obtained in the case of one rabbit, the percentage of positive results is 26·2 per cent., or with the above-named cultures 28·7 per cent., as against 66 per cent. in the case of 30 organs tested by Ford. It is difficult to determine whether the bacteria found were really contained in the organs or not, although every care was taken to avoid contamination. It is well known that organisms of the types detected are ubiquitous in the air and it is quite possible that the germs or their spores adhered to the moist surfaces of the exposed organs during the post-mortem examination. By way of settling this point a certain number of the animal organs were sterilised in the autoclave at a temperature of 128°C. for 20 minutes. This effectually destroyed any bacteria or their spores that might happen to be present either inside or on the surface of the organs. The organs were then placed in sterile Petri dishes in the neighbourhood of an animal on which a post-mortem examination was about to be made and exposed to the air for five or ten minutes. They were next passed through the flame, placed in broth, and incubated at blood heat for two days. In short, they were subjected to exactly the same treatment as the fresh organs in the former experiments. In the case of the organs which had been exposed for ten minutes to the air of the post-mortem room only one out of five proved sterile, whilst of those exposed for five minutes only one out of seven organs was found to be sterile. The organisms found were similar to those met with in the previous experiments—viz., staphylococci and non-

pathogenic saprophytic bacilli. From these experiments with autoclaved organs the presumption is strong that the organisms detected in the previous experiments were not really contained within the tissues but that they had, in spite of the precautions taken, fallen on the surface of the organs during some part of the manipulations. In Ford's experiments the frequent opening of the test-tubes may have led to similar contamination.

It is rather a significant fact that in my experiments no growth was obtained from the spleen, which was always removed first, while the mesenteric glands, which were removed last, gave the highest percentage of positive results—viz., seven out of ten glands tested. It might, of course, be suggested that the mesenteric glands form the natural channel for germs passing out of the intestine and that their presence in these glands could thus be accounted for. An injection of five cubic centimetres of broth culture of the organisms intraperitoneally into the same species of animal gave rise to no apparent symptoms or even temporary illness. The following table gives the results of the broth cultivations from the organs of ten rabbits :—

No. rabbit.	Liver.	Heart.	Right kidney.	Left kidney.	Spleen.	Mesenteric glands.	Suprarenal.	Peritoneal fluid.	Pathogenicity.
1	—	—	—	—	—	C. & B.	—	—	—
2	—	—	—	—	—	—	—	B.	—
3	—	—	B.	—	—	B.	B.	—	—
4	B.	—	B.	—	—	B.	—	—	—
5	—	B.	—	B.	—	—	—	B.	—
6	—	—	—	C.	—	B.	—	B.	—
7	C.	—	—	C.	—	B.	C.	B.	—
8	—	—	—	—	—	B.	—	C.	—
9	B. coli.	—	—	—	—	B. coli.	—	C.	B. coli + C. –
10	—	—	—	—	—	—	—	—	—

B. = bacillus C. = coccus.

These results lead to the following conclusions: 1. That normal organs do not contain pathogenic bacteria. 2. That the presence of non-pathogenic organisms in the cultures is probably due to accidental contamination. These conclusions coincide with those arrived at by Meissner, Hauser, Neisser, Welch, and Opitz. After the above experiments were finished my attention was drawn to an article by A. Wrzosek[2] who found that on adding cultures of the bacillus prodigiosus, bacillus fluorescens, bacillus violaceus, and bacillus pyocyaneus to the food of certain animals and killing these animals from five to 20 days afterwards, these organisms were found in the mesenteric glands, lungs, liver, muscles, and spleen in from 30 to 50 per cent. of the animals. Wrzosek accounts for the small percentage of animals in which they were found by the supposition that the phagocytes destroy organisms in a very short time.

[1]The Bacteriology of Normal Organs, Journal of Hygiene, 1901.
[2]A. Wrzosek: Bulletin de l'Académie des Sciences de Cracovie, November, 1903.

160. Arthur Harden und William John Young: Gährversuche mit Presssaft aus obergähriger Hefe.

(Eingegangen am 8. Februar 1904.)

Die bisher über die alkoholische Gährung mit dem ausgepressten Saft obergähriger Hefe angestellten Versuche schienen zu zeigen, dass zwischen diesem Material und den aus untergährigen Hefen gewonnenen Presssäften gewisse Unterschiede bestehen, und liessen auch einige interessante Fragen bezüglich des Verlaufs der Gährung noch unbeantwortet. Die wichtigste Untersuchung über den Presssaft aus obergähriger Hefe ist die von Macfadyen, Morris und Rowland[1]); die Hauptpunkte, in welchen ihre Resultate von denen Buchner's[2]) abweichen, der mit dem Presssaft aus untergähriger Hefe arbeitete, lassen sich etwa wie folgt kurz zusammenfassen: 1) Die Selbstgährung des Presssaftes war häufig, aber nicht durchgängig, grösser als die Gährung bei Zusatz von Zucker, während nach Buchner die Selbstgährung nur etwa 10 pCt. der Zuckergährung ausmacht. — 2) Mässige Verdünnung mit Wasser oder physiologischer Kochsalzlösung vernichtete die Gährkraft des Presssaftes nahezu völlig. — 3) Nur bei einer sehr lebhaften Gährung wurden Kohlendioxyd und Alkohol in dem für die gewöhnliche alkoholische Gährung charakteristischem Verhältniss aus dem Zucker erzeugt.

Zu diesen Abweichungen trat noch die später von Buchner bestätigte Eigenthümlichkeit hinzu, dass wenn man den Presssaft auf Zucker einwirken liess, der Verlust an diesem Kohlehydrat grösser war, als dem Betrag an entstehendem Kohlendioxyd und Alkohol entsprochen hätte.

Die vorliegende Arbeit wurde in erster Linie in der Absicht unternommen, unsere Kenntnisse bezüglich des Presssaftes aus der obergährigen Hefe zu erweitern, unter specieller Berücksichtigung der

[1]) Proc. Roy. Soc. 64, 280 [1900]; diese Berichte 33, 2764 [1900].
[2]) Die Zymasegährung. München und Berlin, 1903.

jenigen Punkte, in welchen dieses Material sich von dem Presssaft aus untergähriger Hefe zu unterscheiden schien; ausserdem aber bemühten wir uns, eine bessere Einsicht in jene chemischen Veränderungen zu gewinnen, welche die Zersetzung des Zuckers durch Hefepresssaft begleiten, bezw. durch diese veranlasst werden.

I. Darstellung des Hefepresssaftes.

Die aus der Brauerei kommende Hefe wurde abgesaugt und in einer kleinen Filterpresse gut mit verdünnter Salzlösung ausgewaschen; dann wurde sie in einer hydraulischen Presse trocken gepresst. Nach dem Vermischen mit dem gleichen Gewicht reinen weissen Sandes wurden hierauf die Zellwände durch 2—3-stündiges Zerreiben in dem von Rowland[1]) beschriebenen Desintegrator zerrissen. Schliesslich wurde die zerriebene Masse mit gut ausgeglühter Kieselguhr zu einer dicken Paste angerieben, die in ein Tuch eingehüllt und in einer hydraulischen Presse (Rowland, l. c.) ausgedrückt wurde. Der herausquellende Saft wurde alsdann behufs Entfernung von Zellen, welche beim Pressen durch das Tuch gegangen waren, centrifugirt.

Während des Zerreibens wurde die Masse kühl erhalten durch Umgeben des Gefässes mit einem Schlangenrohr, durch welches flüssiges Kohlendioxyd hindurch verdampfte; in anderen Fällen wurde die Kühlung mittels eines Stromes von kaltem Wasser bewirkt.

II. Inactive Presssäfte.

Im Laufe unserer Versuche wurden gelegentlich — gewöhnlich in den Sommermonaten — auch ganz inactive Presssäfte erhalten, obwohl weder im Material noch in den angewendeten Methoden irgend eine Veränderung vorgenommen wurde. So erwies sich z. B. ein am 25. Juni 1903 dargestellter Presssaft als inactiv, während unter anscheinend völlig identischen Bedingungen am 29. des gleichen Monats ein normal wirksamer Presssaft gewonnen wurde.

III. Versuchsanordnung.

Das sich bei der Selbstgährung des Presssaftes oder bei seiner Einwirkung auf Zucker entwickelnde Kohlendioxyd wurde auf einem der beiden folgenden Wege ermittelt:

1. *Gravimetrische Methode.* Die Gährung vollzog sich in einem kleinen Erlenmeyer'schen Kölbchen; dasselbe trug ein gebogenes Rohr, welches in concentrirte Schwefelsäure eintauchte, die sich

[1]) Journ. Physiol. 27, 53 [1901].

in einem zweiten ähnlichen Kölbchen befand. Das Ganze wurde dann gewogen, der Apparat mit einem Chlorcalciumrohr verbunden und die Kölbchen in einem Brutschrank die erforderliche Zeit auf 25° erwärmt. Hierauf wurde ein von Feuchtigkeit und Kohlendioxyd befreiter Luftstrom 20 Minuten über die Flüssigkeit und durch die Schwefelsäure geleitet und das Ganze wiederum gewogen. Der sich hierbei ergebende Gewichtsverlust entsprach der bei der Gährung in Freiheit gesetzten Menge Kohlendioxyd.

Für diese Versuche diente Thymol als Antisepticum, da sich Toluol in Folge seiner Flüchtigkeit nicht verwenden liess. Hierbei stellte sich jedoch heraus, dass Thymol nicht so gut antiseptisch wirkt wie Toluol, denn in einigen wenigen Fällen zeigte es sich, dass Hefezellen und andere Mikroorganismen in der Flüssigkeit selbst bei Gegenwart von Thymol zur Entwickelung gelangt waren. In Folge dessen wurde der Presssaft nach der Gährung bei jedem Versuch mikroskopisch geprüft und das Resultat verworfen, sobald sich irgend welches Wachsthum von Mikroorganismen erkennen liess.

2. *Volumetrische Methode.* Das in diesem Falle angewendete Verfahren war das von Macfadyen, Morris und Rowland (loc. cit.) beschriebene. Als Antisepticum diente stets Toluol. Das sich entwickelnde Kohlendioxyd strich durch 50 ccm Natronlauge. Nach beendigter Gährung wurde 20 Min. lang kohlensäurefreie Luft über die Flüssigkeit und durch das Alkali geleitet. Das die Natronlauge enthaltende Gefäss wurde dann in ein 100 ccm-Kölbchen entleert und mit kohlensäurefreiem Wasser gut ausgespült; hierauf wurde bis zur Marke aufgefüllt. Schliesslich wurde die alkalische Flüssigkeit zunächst mit Normalsäure bis zur Neutralität gegen Phenolphtaleïn und dann mit $^1/_{10}$-Normalsäure bis zur Neutralität gegen Methylorange titrirt. Aus der Differenz zwischen den Resultaten dieser beiden Titrationen ergab sich der Gehalt an Kohlendioxyd.

Es liess sich feststellen, dass praktisch die Gesammtmenge des Kohlendioxydes durch 20 Min. langes Ueberleiten von Luft über die Oberfläche der kalten Flüssigkeit in das Alkali übergeführt wurde. Bei einem Versuch ergaben sich auf diesem Wege 0.229 g Kohlendioxyd, während 0.235 g erhalten wurden, als man die Luft über die Oberfläche der auf 100° erwärmten Flüssigkeit leitete.

IV. Zusatz von verschiedenen Zuckerarten.

Bei allen Versuchen wurde Glucose als zu vergährender Zucker gewählt. Auf Grund einer gewissen Zahl von Experimenten (Tabelle I) liess sich feststellen, dass Glucose und die äquivalente Menge Rohrzucker praktisch die gleichen Resultate ergaben.

Tabelle I.

Vergleich der Rohrzucker- und Glucose-Vergährung.

(25 ccm Presssaft, 25°.)

(No. 1—6 wurden mit Toluol, No. 7—10 mit Thymol ausgeführt.)

No.	Datum	Hinzugefügter Zucker	Kohlendioxyd in g	
			24 Stdn.	48 Stdn.
1	17. IV. 03	2.5 g Glucose	—	0.31
2	do.	2.3 g Rohrzucker	—	0.31
3	16. V. 03	2.5 g Glucose	—	0.30
4	do.	2.3 g Rohrzucker	—	0.29
5	17. V. 03	2.5 g Glucose	—	0.44
6	do.	2.3 g Rohrzucker	—	0.45
7	25. XI. 02	10 g Glucose	0.16	0.20
8	do.	9.3 g Rohrzucker	0.14	0.19
9	25. II. 03	10 g Glucose	0.47	0.49
10	do.	9.3 g Rohrzucker	0.49	0.51

V. Selbstgährung und Glucose-Vergährung.

Behufs Feststellung des allgemeinen Charakters der Selbstgährung und der Glucose-Vergährung wurde eine Reihe von Versuchen mit wechselnden Mengen Glucose durchgeführt, deren Resultate in den Tabellen II—IV niedergelegt sind.

Tabelle II.

Vergleich der Selbstgährung mit der Glucose-Vergährung.

(25 ccm Presssaft, Thymol, 25°.)

No.	Datum	Aus 25 ccm Presssaft entwickeltes Kohlendioxyd in g							
		Selbtgährung nach Stdn.				+ 2.2 g Glucose nach Stdn.			
		24	48	72	96	24	48	72	96
11	30. IV. 01	0.17	0.21	0.21	—	0.25	0.30	0.32	—
12	8. V. 01	0.06	0.09	0.13	—	0 08	0.10	0.19	—
13	6. XI. 02	—	0.14	—	0.16	—	0.61	—	0.66
14	13. XI. 02	—	0.04	—	0.06	—	0.42	—	0.46
15	25. XI. 02	—	—	0.10	—	—	—	0.48	—
	do.	—	—	0.11	—	—	—	0.49	—
16	13. I. 03	—	—	0 09	—	—	—	0.25	—
17	21. I. 03	—	—	0.10	—	—	—	0.29	—

Tabelle III.

Vergleich der Selbstgährung mit der Glucose-Vergährung.

(25 ccm Presssaft, Toluol, 25°.)

No.	Datum	Kohlendioxyd in g			
		Selbstgährung nach Stdn.		+ 2.5 g Glucose nach Stdn.	
		48	72	48	72
18	7. V. 03	0.04	—	0.31	—
19	11. V. 03	0.18	—	0.30	—
20	13. V. 03	0.25	—	0.44	—
21	15. V. 03	—	0.32	—	0.49
22	18. V. 03	0.19	—	0.37	—
23	19. V. 03	0.23	—	0.75	—
24	20. V. 03	0.29	—	0.50	—
25	25. V. 03	0.11	—	0.48	—
26	27. V. 03	0.09	—	0.50	—
27	3. VI. 03	0.06	—	0.20	—
28	5. VI. 03	—	0.29	—	0.60
29	9. VI. 03	0.04	—	0.35	—
30	29. VI. 03	0.04	—	0.31	—
31	6. VII. 03	0.31	—	0.26	—

Tabelle IV.

Vergleich der Selbstgährung mit der Glucose-Vergährung.

(25 ccm Presssaft, Thymol, 25°.)

No.	Datum	Kohlendioxyd in g							
		Selbstgährung nach Stdn.				+ 10 g Glucose nach Stdn.			
		24	48	72	96	24	48	72	96
32	20. VI. 01	—	0.13	—	—	—	0.33	—	—
	do.	—	0.15	—	—	—	0.37	—	—
33	24. VI. 01	0.08	0.09	0.09	—	0.15	0.19	0.20	—
34	10. VII. 01	0.09	0.13	—	—	0.12	0.15	—	—
35	7. X 01	0.08	0.11	—	—	0.22	0.26	—	—
36	15. X. 01	0.03	0.04	—	—	0.14	0.16	—	—
37	17. X. 01	0.16	0.20	—	0.21	0.16	0.22	—	0.24
38	22. X. 01	0.07	0.08	0.09	—	0.26	0.30	0.31	—
39	14. XI. 01	0.07	—	—	0.11	0.05	—	—	0.09
40	18. XI. 01	0.07	—	—	0.09	0.08	—	—	0.11
41	25. XI. 01	—	0.08	—	—	—	0.10	—	—
42	5. XII. 01	0.13	—	—	—	0.10	—	—	—
43	8. I. 02	0.10	0.13	0.15	—	0.18	0.21	0.23	—
44	27. III. 03	0.20	—	0.37	—	0.55	—	0.72	—

Auf Grund der voranstehend mitgetheilten Ziffern lassen sich folgende Schlussfolgerungen ziehen:

1. Der durchschnittliche Betrag der Zuckervergährung ist unverkennbar geringer als der von Buchner bei den Presssäften aus untergähriger Hefe beobachtete, er entspricht aber den Angaben von Macfadyen, Morris und Rowland über den Presssaft aus obergähriger Hefe.

2. Der Betrag der Selbstgährung ist sehr veränderlich: er schwankt zwischen 0.02—0.32 g Kohlendioxyd aus 25 ccm Presssaft. Dem absoluten Werthe nach entspricht er den von Buchner (0.10—0.45 g), wie auch den von Macfadyen, Morris und Rowland (0.06—0.495 g) gegebenen Zahlen. Da der Umfang der durch unseren Presssaft hervorgerufenen Zuckervergährung im Vergleich zu der von Buchner beobachteten nur gering ist, macht die Selbstgährung einen erheblichen Bruchtheil der Gesammtgährung bei Gegenwart von Zucker aus. In zwei Fällen (vergl. Versuch Nr. 31 der Tabelle III und Versuch Nr. 39 der Tabelle IV) war die Selbstgährung etwas grösser als die Gesammtgährung; in mehreren anderen Fällen kam sie derselben fast gleich. Kein einziger der von uns untersuchten Presssäfte wies jedoch in dieser Richtung so grosse Differenzen auf, wie sie in einigen Fällen bei Macfadyen, Morris und Rowland zu Tage getreten sind.

Bei dem Vergleich der im Voranstehenden gegebenen Zahlen mit denen von Buchner fällt es sofort auf, dass bei dem von uns erhaltenen Presssaft der Betrag der in den ersten 24 Stunden eintretenden Gesammtgährung erheblich grösser ist, als bei den Presssäften aus untergähriger Hefe, mit welchen Buchner arbeitete. Letzterer hat gewöhnlich gefunden, dass die Gesammtgährung in 72—96 Stunden 1.5—3 Mal grösser war, als die in den ersten 24 Stunden eintretende; doch ergaben sich hierbei gelegentlich auch Ausnahmen (vergl. Zymasegährung, S. 83, 94, 95). Bei den von uns benutzten Presssäften war andererseits die mit der gleichen Zuckerconcentration erhältliche Gesammtgährung selten grösser als der $1^1/_2$-fache Betrag der in den ersten 24 Stunden zu beobachtenden; gewöhnlich war er sogar erheblich kleiner. Dies ist, wie auch schon Buchner vermuthete, vielleicht dem Umstande zuzuschreiben, dass obergährige Hefen weniger Zymase enthalten als untergährige, oder aber auf gewisse Unterschiede zurückzuführen, welche die beiden Hefearten in ihrem Gehalt an proteolytischen Enzymen aufweisen.

VI. Ohne Verwendung von Kieselguhr dargestellte Presssäfte.

In der Absicht, festzustellen, ob die beim Auspressen des Hefepresssaftes bisher benutzte Kieselguhr vielleicht mit Vortheil durch

ein anderes Material ersetzt werden könnte, wurden einige wenige Versuche angestellt, bei welchen die zerriebene, aus Hefe und Sand bestehende Masse mit ausgepresster Hefe vermischt und dann nochmals gepresst wurde. Der so gewonnene und centrifugirte Presssaft besass ganz die gleichen Eigenschaften wie der mit Kieselguhr dargestellte, wie die in der Tabelle V zusammengestellten Resultate zeigen.

Tabelle V.

Ohne Verwendung von Kieselguhr dargestellter Presssaft.

(25 ccm Presssaft, Thymol, 25°.)

No.	Datum	Zugesetze Glucose in g	Kohlendioxyd in g		
			24 Stdn.	48 Stdn.	72 Stdn.
45	13. VI. 02	—	—	—	0.11
46	do.	2.2	—	—	0.20
47	20. VI. 02	—	0.03	—	0.04
48	do.	10	0.10	—	0.14
49	24. VI. 02	—	0.02	—	—
50	do.	2.5	0.10	—	—
51	27. VI. 02	—	—	—	0.11
52	do.	1	—	—	0.16
53	15. VII. 02	—	—	0.02	0.04
54	do.	1.1	—	0.09	0.10
55	30. V. 02	—	0.14	—	—
56	do.	10	0.23	—	—

VII. Glykogen-Vergährung.

Die Frage, ob das Glykogen der Hefezelle von dem Presssaft schneller vergohren wird als die Glucose, wurde durch eine Reihe von Versuchen zu lösen versucht, bei welchen unter Berücksichtigung der Selbstgährung, diejenigen Mengen von Kohlendioxyd ermittelt wurden, die sich bei der Vergährung von Glucose und der äquivalenten Menge reinen Hefe-Glykogens[1]) ergaben.

Aus diesen Zahlen lässt sich ersehen, dass die Glykogenvergährung sich unverkennbar weniger vollständig vollzieht als die Zuckervergährung, wenn sie Letztere auch in einem Falle (Versuch No. 77) überflügelte. Aehnliche Resultate hat übrigens auch Buchner (Zymasegährung, S. 102) mit einem von Merck bezogenen Glykogen erhalten. In allen Fällen war jedoch die Glykogenvergährung stärker als die Selbstgährung.

[1]) Harden und Young, Transact. chem. Soc. 81, 1224 [1902].

Tabelle VI.

Vergährung von Glykogen.

(25 ccm Presssaft, 25°; bei den Versuchen No. 72—74 diente Toluol, bei den übrigen Thymol als Antisepticum.)

No.	Datum		Kohlendioxyd in g			
			24 Stdn.	48 Stdn.	72 Stdn.	96 Stdn.
57	13. VI. 02	Selbstgährung	—	—	0.11	—
58	do.	+ 2.2 g Glucose	—	—	0.20	—
59	do.	+ 2 g Glykogen	—	—	0.16	—
60	6. XI. 02	Selbstgährung	—	0.14	—	0.16
61	do.	+ 2.2 g Glucose	—	0.61	—	0.66
62	do.	+ 2 g Glykogen	—	0.29	—	0.33
63	13. XI. 02	Selbstgährung	—	0.04	—	0.06
64	do.	+ 2.2 g Glucose	—	0.42	—	0.46
65	do.	+ 2 g Glykogen	—	0.15	—	0.18
66	13. I. 03	Selbstgährung	—	—	0.09	—
67	do.	+ 2.2 g Glucose	—	—	0.25	—
68	do.	+ 2 g Glykogen	—	—	0.12	—
69	24. VI. 02	Selbstgährung	0.02	—	—	—
70	do.	+ 2.5 g Glucose	0.10	—	—	—
71	do.	+ 2.25 g Glykogen	0.08	—	—	—
72	6. VII. 02	Selbstgährung	—	0.03	—	—
73	do.	+ 2.5 g Glucose	—	0.26	—	—
74	do	+ 2.25 g Glykogen	—	0.16	—	—
75	27. VI. 02	Selbstgährung	—	—	0.11	—
76	do.	+ 1 g Glucose	—	—	0.16	—
77	do.	+ 0.9 g Glykogen	—	—	0.18	—

VIII. Verhältniss des sich bei der Zuckervergährung mittels Hefepresssaft bildenden Alkohols zum Kohlendioxyd.

Die Bestimmung des Kohlendioxyds ist schon weiter oben beschrieben worden. Der Alkoholgehalt wurde sowohl in dem ursprünglichen Presssaft, als auch in der nach der Gährung resultirenden Flüssigkeit ermittelt. Die Differenz entsprach dann der während der Gährung entstehenden Menge Alkohol.

Der Versuch wurde so ausgeführt, dass 25 ccm Presssaft mit einer gewogenen Menge Glucose und etwas Thymol angesetzt wurden, und zwar in einem kleinen Erlenmeyer-Kolben, der mit Hülfe eines gebogenen Rohrs mit einem zweiten Kölbchen in Verbindung stand. Letzteres enthielt etwas Wasser, durch welches der von den entweichenden Gasen mitgeführte Alkohol zurückgehalten wurde. Nach beendigter Gährung wurde der Apparat gut ausgewaschen und die Flüssigkeit auf 150 ccm aufgefüllt. Dann wurden 100 ccm abdestillirt;

nach Zusatz von etwas Pottasche, welche das Thymol und flüchtige Säuren zurückhalten sollte, wurden von diesem Destillat 50 ccm übergesiedet und aus dem specifischen Gewicht des so gewonnenen Condensats der Alkoholgehalt berechnet. Der ursprünglich im Presssaft vorhandene Alkohol wurde in 25 ccm Saft in genau derselben Weise ermittelt.

Die Einzelheiten eines solchen Versuchs ergaben sich beispielsweise aus Folgendem:

Versuch No. 81. 25 ccm Presssaft + 2 g Glucose (+ Thymol).

In 25 ccm Presssaft ursprünglich vorhandener Alkohol . . .	0.76 g
» » nach der Gährung » » . . .	1.18 »
Zunahme	0.42 g
Kohlendioxyd aus 25 ccm Presssaft + 2 g Glucose	0.46 g.

Tabelle VII.

Verhältniss des entstehenden Alkohols zum Kohlendioxyd.

(25 ccm Presssaft, Thymol, 25°.)

No.	Datum	Zugesetzte Glucose	Zeit	CO_2	Zunahme an Alkohol	Alkohol : Kohlendioxyd, ber. = 1.04
		g	Stdn.	g	g	g
78	30. IV. 01	0	48	0.21	0.19	0.90
79	»	2.5	48	0.32	0.36	1.12
80	8. V. 01	2.5	72	0.19	0.18	0.95
81	2. III. 03	2	60	0.46	0.42	0.91
82	24. III. 03	2	60	0.60	0.56	0.92

Die Ziffern der Tabelle VII lassen erkennen, dass die von uns dargestellten Presssäfte sowohl bei der Selbstgährung als auch bei der Zuckervergährung stets annähernd gleiche Mengen von Kohlendioxyd und Alkohol liefern, und zwar scheint dieses Verhältniss davon unabhängig zu sein, ob die Gährung mehr oder weniger lebhaft verläuft, da es auch bei zwei Presssäften zu Tage trat, von welchen der eine nur 0.19 g Kohlendioxyd, der andere dagegen 0.6 g Kohlendioxyd (aus 25 ccm) erzeugte. Auch bei der Selbstgährung findet sich (vergl. Versuch No. 78) das gleiche Verhältniss wieder.

Buchner (Zymasegährung, S. 210) hat beim Rohrzucker ebenfalls annähernde Gleichheit in den Mengenverhältnissen zwischen Alkohol und Kohlendioxyd festgestellt; Macfadyen, Morris und Rowland erhielten ein ähnliches Resultat dagegen nur dann, wenn die Gährung eine sehr energische gewesen war.

IX. Einfluss der Verdünnung.

Die Wirkung des Verdünnens wurde in einer grossen Zahl von Fällen verfolgt, da Macfadyen, Morris und Rowland auf Grund eines kleineren Versuchsmaterials zu der Ansicht gelangt sind, dass

sowohl die Selbstgährung wie auch die Zuckervergährung fast vollständig durch den Zusatz von 1—2 Vol. Wasser zum Stillstand kommt. Andererseits hat Buchner nur eine geringfügige Verminderung der Zuckervergährung feststellen können, als er seinen Presssaft mit Wasser oder Zuckerlösung verdünnte. Den Einfluss der Verdünnung auf die Selbstgährung hat er nicht studirt.

Tabelle VIII.

Einfluss der Verdünnung mit Wasser auf die Selbstgährung.

(25 ccm Hefepresssaft + n Vol. Wasser + Toluol; 25°).

No.	Datum	Kohlendioxyd nach 48 Stdn.			
		n = 0	n = 2	n = 3	n = 6
83	7. V. 03	0.04	0.05	0.05	—
84	11. V. 03	0.18	0.16	0.16	—
85	13. V. 03	0.25	0.21	0.19	—
86	15. V. 03	0.32	0.30	0.31	—
87	18. V. 03	0.19	0.18	—	0.15
88	19. V. 03	0.23	—	0.22	—
89	20. V. 03	0.29	0.29	0.28	—
90	25. V. 03	0.11	0.11	0.11	—
91	27. V. 03	0.09	0.09	0.09	—
92	3. VI. 03	0.06	—	0.06	—
93	5. VI. 03	0.29	—	0.29	—
94	9. VI. 03	0.04	—	0.04	—

Tabelle IX.

Einfluss der Verdünnung mit Wasser auf die Zuckervergährung.

(25 ccm Hefepresssaft + n Vol. Wasser + Toluol: 25°.)

No.	Datum	Vol. Wasser n	Glucose g	Kohlendioxyd nach 72 Stdn.
95	18. II. 03	0	2	0.55
96	do.	1	2	0.23
97	do.	2	2	0.33
98	do.	3	2	0.25
99	19. III. 03	0	2	0.44
100	do.	1	2	0.35
101	do.	2	2	0.30
102	do.	3	2	0.28
103	24. III. 03	0	2	0.60
104	do.	1	2	0.43
105	do.	3	2	0.44
106	27. IV. 03	1	2.5	0.51
107	do.	3	2.5	0.38
108	31. III. 03	0	10	0.49
109	do.	1	10	0.45
110	do.	3	10	0.36

Tabelle X.

Einfluss der Verdünnung mit Zuckerlösung auf die Zuckervergährung.

(25 ccm Hefepresssaft + n Vol. Wasser + 2.2 g Glucose auf je 25 ccm Flüssigkeit; bei den Versuchen No. 111—121, 124—125 diente Thymol, bei No. 122—123 und 126—129 Toluol als Antisepticum; 25°.)

No.	Datum	Vol. Wasser n	Kohlendioxyd nach			
			48 Stdn.	72 Stdn.	96 Stdn.	108 Stdn.
111	13. XI. 02	0	0.42	—	0.46	—
112	do.	1	0.36	—	0.41	—
113	do.	2	0.26	—	0.37	—
114	6. XI. 02	0	0.61	—	0.66	—
115	do.	1	0.44	—	0.54	—
116	25. XI. 02	0	—	0.49	—	—
117	do.	1	—	0.50	—	—
118	13. I. 03	0	—	0.25	—	—
119	do.	1	—	0.25	—	—
120	21. I. 03	0	—	0.29	—	—
121	do.	1	—	0.26	—	—
122	4. II. 03	0	—	0.34	—	—
123	do.	1	—	0.35	—	—
124	do.	0	—	0.35	—	—
125	do.	1	—	0.31	—	—
126	11. II. 03	0	—	—	—	0.56
127	do.	1	—	—	—	0.45
128	do.	2	—	—	—	0.42
129	do.	3	—	—	—	0.60

Die Tabellen 8—11 (vergl. S. 1063) zeigen den Einfluss, welchen die Verdünnung mit Wasser auf die Selbstgährung und die Verdünnung mit Zuckerlösung oder reinem Wasser auf die Zuckervergährung ausüben. Die Wirkung des Verdünnens auf die Selbstgährung erwies sich bei allen diesen Versuchen als sehr unbedeutend: der Zusatz von 3 und selbst 6 Vol. Wasser übte nur einen sehr geringen Einfluss auf die Menge des entwickelten Kohlendioxyds aus (Tabelle VIII). Beträchtlicher ist dagegen die Wirkung des Verdünnens mit Wasser auf glucosehaltige Presssäfte; hier verringerte ein Zusatz von 3 Vol. Wasser die Gährung auf ungefähr 0.5—0.7 ihres ursprünglichen Betrages (Tabelle IX). Schliesslich sei noch darauf aufmerksam gemacht, dass Verdünnen mit Zuckerlösung, gleichgültig, ob dieselbe 2.2 g oder 10 g Glucose in 25 ccm Wasser enthält (Tabelle X und XI), regelmässig nur eine sehr geringe Verminderung, in einigen Fällen sogar eine mässige Steigerung der Gährung veranlasst.

In keinem Falle brachte das Verdünnen die Gährung zum Stillstand.

Tabelle XI.

Einfluss der Verdünnung mit Zuckerlösung auf die Zuckervergährung.

(25 ccm Hefepresssaft + n Vol. Wasser + 10 g Glucose auf je 25 ccm Flüssigkeit + Thymol; 25°.)

No.	Datum	Vol. Wasser n	Kohlendioxyd nach			
			24 Stdn.	48 Stdn.	72 Stdn.	96 Stdn.
130	7. X. 01	0	0.22	0.26	—	—
131	do.	1	0.26	0.27	—	—
132	15. X. 01	0	0.14	0.16	0.16	—
133	do.	1	0.13	0.16	0.17	—
134	do.	2	0.09	0.13	0.14	—
135	17. X. 01	0	0.16	0.22	—	0.24
136	do.	1	0.21	0.29	—	0.34
137	do.	2	0.17	0.26	—	0.32
138	do.	4	0.10	0.21	—	0.30
139	do.	6	0.07	0.17	—	—
140	22. X. 01	0	0.26	0.30	0.31	—
141	do.	1	0.29	0.32	0.34	—
142	do.	2	0.22	0.29	0.31	—
143	do.	4	0.25	0.32	0.35	—
144	do.	6	0.18	0.25	0.28	—
145	18. XI. 01	0	0.08	—	0.11	—
146	do.	2	0.07	—	0.10	—
147	do.	3	0.07	—	0.11	—
148	25. XI. 01	0	—	0.10	—	—
149	do.	1	—	0.12	—	—
150	do.	2	—	0.09	—	—
151	do.	5	—	0.11	—	—
152	8. I. 02	0	0.18	0.21	0.23	—
153	do.	1	0.15	0.15	0.19	—
154	do.	2	0.11	0.15	0.19	—
155	do.	5	0.12	0.15	0.21	—

X. Verhältniss des verbrauchten Zuckers zum entstehenden Alkohol und Kohlendioxyd.

Von Macfadyen, Morris und Rowland ist beobachtet worden, dass diejenige Menge von Zucker, die im Verlauf der Gährung vom Hefepresssaft aufgebraucht wird, grösser ist als der Betrag an entstehendem Alkohol und Kohlendioxyd. Später hat dann auch Buchner gefunden, dass, wenn eine Rohrzuckerlösung so vollständig wie möglich mit »Aceton-Dauerhefe« vergohren wird, die Summe an Alkohol und Kohlendioxyd um rund 15 pCt. kleiner ist als die aus der Gesammtmenge des Zuckers sich berechnende Quantität.

Die Schwierigkeiten, denen Buchner bei der Bestimmung des Zuckers in Gegenwart von Presssaft begegnete, lassen sich durch Anwendung der Pavy'schen Methode (Titration mit ammoniakalischer

Kupferlösung) vermeiden, die zur Anwendung auf derartige Flüssigkeiten ganz besonders gut geeignet ist.

Unsere ersten Versuche dienten dazu, den Betrag der Differenz zwischen dem verbrauchten Zucker und der Summe der gefundenen Producte aufzuklären. Zu diesem Zwecke wurde folgende Versuchsanordnung inne gehalten: 25 ccm des frischen Presssaftes wurden auf 100 ccm verdünnt und das Reductionsvermögen nach der Pavy'schen Methode festgestellt. Hierbei sei bemerkt, dass der Presssaft stets reducirenden Zucker enthielt, dessen durchschnittliche Menge ungefähr 0.8 g Glucose in 100 ccm entsprach. Zu weiteren 25 ccm des ursprünglichen Presssaftes wurde dann eine gewogene Menge Glucose hinzugegeben, die Vergährung wie gewöhnlich ausgeführt und das entstehende Kohlendioxyd ermittelt. Hiernach wurde der Betrag an thatsächlich vergohrenem Zucker umgerechnet in äquimolekulare Mengen Kohlendioxyd und Alkohol. Dies ist zulässig, nachdem Buchner gezeigt hat, dass die übrigen, von lebender Hefe bei der Gährung noch erzeugten Producte hier in nennenswerthem Betrage nicht auftreten.

Der gleiche Presssaft wurde dann auf 100 ccm verdünnt und sein Reductionsvermögen wie weiter oben beschrieben bestimmt. Die Differenz zwischen dem so gewonnenen Resultat und der vor Beginn der Gährung ermittelten Zahl + dem Betrage an zugesetzter Glucose ergiebt die Menge der thatsächlich vorhandenen Glucose; des weiteren entspricht die Differenz zwischen dem sich so ergebenden Werth und dem aus dem Gewicht des sich bildenden Kohlendioxyds berechneten dem Betrage an nicht in Rechnung gesetztem bezw. bei dem Versuch verlorengegangenem Zucker.

Tabelle XII (S. 1065) bringt die in einer Reihe von Fällen, und zwar sowohl bei unverdünnten wie auch bei mit Zuckerlösung verdünnten Presssäften erhaltenen Resultate. Die Fehler der einzelnen Bestimmungen waren hierbei niemals grösser als 2 pCt. der Gesammtmenge des vorhandenen Zuckers, blieben aber meistens unter 1 pCt. Der Verlust an Zucker schwankte bei den einzelnen Versuchen beträchtlich und war gewöhnlich, aber nicht durchgängig, ein grösserer, wenn der Presssaft mit Zuckerlösung verdünnt wurde. Zwischen dem Gesammtbetrage an vorhandenem und an zu Kohlendioxyd und Alkohol vergohrenem Zucker liess sich keine bestimmte Beziehung feststellen. Die Versuche No. 177 und 179 wurden doppelt ausgeführt und zwar unter Verwendung von Toluol wie auch von Thymol als Antisepticum; die Resultate stimmen befriedigend überein. Bei einer kleinen Zahl von Versuchen (Tabelle XIII) wurde sowohl das Kohlendioxyd als auch der entstehende Alkohol bestimmt und die Summe dieser beiden als Gewicht des thatsächlich vergohrenen Zuckers aufgefasst. Auch hier

macht der Verlust an Zucker einen beträchtlichen Bruchtheil (14—36 pCt.) des gesammten, zur Vergährung gelangten Zuckers aus.

Tabelle XII.

Beziehung zwischen verbrauchtem Zucker und den entstehenden Producten.

(25 ccm Presssaft; bei den Versuchen No. 172—177 und 179 diente Toluol bei den übrigen Thymol als Antisepticum).

No.	Datum	Zeit	Vol. des zugefügten Wassers	Zusatz von Glucose in g	aus dem Kohlendioxyd berechneter Zucker	verbrauchter Zucker	Verlust an Zucker
156	13. XI. 02	88 Stdn.	0	2.2	0.92	1.13	0.21
157	do.	do.	1	4.4	0.82	1.57	0.75
158	do.	do.	2	6.6	0.74	1.30	0.56
159	9. II. 03	108 Stdn.	0	2.2	1.12	1.24	0.12
160	do.	do.	1	4.4	0.90	1.08	0.18
161	do.	do.	2	6.6	0.84	1.05	0.21
162	do.	do.	3	8.8	1.20	2.06	0.86
163	18. II. 03	60 Stdn.	0	2	1.08	1.39	0.31
164	do.	do.	1	4	1.06	1.30	0.24
165	do.	do.	2	6	0.66	0.99	0.33
166	19. III. 03	80 Stdn.	0	2	0.88	0.97	0.09
167	do.	do.	1	4	0.70	0.88	0.18
168	do.	do.	3	8	0.56	0.82	0.26
169	24. III. 03	60 Stdn.	0	2	1.20	1.36	0.16
170	do.	do.	1	4	0.86	1.19	0.33
171	do.	do.	3	8	0.88	1.26	0.38
172	31. III. 03	60 Stdn.	0	10	0.98	2.23	1.25
173	do.	do.	1	20	0.90	3.08	2.18
174	do.	do.	3	40	0.70	3.31	2.61
175	27. IV. 03	64 Stdn.	1	5	1.02	1.37	0.35
176	do.	do.	3	10	0.76	1.31	0.55
177	4. II. 03	64 Stdn.	0	2	0.68	0.92	0.24
178	do.	do.	0	2	0.70	0.95	0.25
179	do.	do.	1	4	0.70	1.22	0.52
180	do.	do.	1	4	0.60	1.05	0 45

Tabelle XIII.

Beziehung zwischen dem verbrauchten Zucker und den entstehenden Producten.

(25 ccm Presssaft, Thymol, 25°).

No.	Datum	Zugesetzte Glucose in g	Kohlendioxyd + Alkohol	Verbrauchter Zucker	Verlust an Zucker
181	30. IV. 01	2.2	0.68	0.93	0.25
182	8. V. 01	2.5	0.37	0.49	0.12
183	2. III. 03	2	0.88	1.02	0.14
	do.	2	0.88	1.04	0.16
184	24. III. 03	2	1.16	1.36	0.20

Da eine gewisse Menge von Zucker bei jeder Gährung verloren ging, wurden zunächst Versuche unternommen behufs Feststellung, ob dieser Zucker etwa in irgend eine Substanz umgewandelt würde, welche durch Hydrolyse mittels Säuren wieder in Zucker zurückverwandelt werden könnte.

Zu diesem Zwecke war es nothwendig, den Presssaft vor und nach der Vergährung zu hydrolysiren und das Reductionsvermögen der hydrolysirten Producte zu ermitteln. Ferner musste das Reductionsvermögen des nicht hydrolysirten Presssaftes, wie schon beschrieben, bestimmt werden. Dies gelang mit Hülfe des folgenden Verfahrens: Bei jedem Versuche wurden 25 ccm Presssaft in Arbeit genommen und auf 100 ccm verdünnt. In 20 ccm dieser Flüssigkeit wurde zunächst das Reductionsvermögen nach Pavy festgestellt; weitere 20 ccm wurden dann mit 20 ccm Normalsalzsäure vermischt, sodass die gesammte Lösung nunmehr $^1/_2$-*n*. Säure enthielt. Hierauf wurde das Kölbchen mit einem Rückflusskühler versehen und 7 Stdn. im siedenden Wasserbade erhitzt, nachdem frühere Versuche gezeigt hatten, dass Glykogen behufs vollständiger Hydrolyse dieser Reactionsbedingungen bedarf.

Zu einem dritten Quantum von wiederum 20 ccm wurde soviel concentrirte Salzsäure hinzugegeben, dass die Concentration der Säure dreifachnormal wurde, und das Ganze dann in derselben Weise 3 Stdn. erhitzt. Nach dem Abkühlen wurde in jedem Fall mit Ammoniak neutralisirt, in geeigneter Weise verdünnt und nunmehr das Reductionsvermögen ermittelt. Da Glucose beim Erhitzen mit Salzsäure in gewissem Grade zersetzt wird, wurden Controllversuche mit dem reinen Zucker ausgeführt behufs Ermittelung der Höhe des Verlustes, den man bei einer derartigen Behandlung verschiedener Zuckermengen erleidet. Gewogene, zwischen 0.1 und 1.2 g schwankende Quantitäten Glucose wurden in je 20 ccm Wasser aufgenommen, diese Lösungen genau wie oben beschrieben mit Salzsäure behandelt und auf ihr Reductionsvermögen geprüft.

Die so erhaltenen Zahlen wurden nebst den ursprünglich verwendeten Zuckermengen in ein Coordinatensystem eingetragen. Hierbei zeigte es sich, dass sowohl die mit $^1/_2$-normaler als auch die mit 3-fach normaler Säure gewonnenen Resultate eine gerade Linie bildeten. Mit anderen Worten: Der Umfang der Zersetzung ist direct proportional der verwendeten Menge Glucose. Es war deshalb möglich, aus den gezeichneten Curven diejenige Quantität Glucose zu entnehmen, welche dem Reductionsvermögen entsprach, das nach der Hydrolyse durch Titration gefunden wurde.

Die Curven wurden auf ihre Richtigkeit geprüft durch Verwendung bekannter Mengen Glucose, Erhitzen mit Salzsäure, wie oben beschrieben, Bestimmen der Reductionswirkung und Berechnen des wirklich vorhandenen Zuckerquantums, dem das gefundene Reductionsvermögen entsprach, mit Hülfe der Curve. Hierbei wurden, wie Tabelle XIV zeigt, sowohl mit reiner Glucose als auch mit Glucose bei Gegenwart von Presssaft zufriedenstellende Resultate erhalten.

Tabelle XIV. Reine Glucose.

Angewandte Glucose:	Gefunden:	Correctur aus der Curve:
	$^1/_2$-normale Säure.	
0.15	0.146	0.15
0.35	0.340	0.348
0.65	0.643	0.655
0.9	0.85	0.87
	$^3/_1$-normale Säure.	
0.15	0.142	0.16
0.3	0.261	0.295
0.7	0.61	0.71

Bei Gegenwart von Presssaft.

Gefunden nach Correctur:

		$^1/_2$-normale Säure:	$^3/_1$-normale Säure:
Glucose	2.2	2.19	2.15
	4.4	4.31	4.43
Glykogen	2.0	1.93	1.85

Die in Tabelle XV (S. 1068) zusammengestellten Ergebnisse lassen erkennen, dass der scheinbar verloren gehende Zucker in Wirklichkeit in der Lösung in einer Form vorhanden ist, welche durch Hydrolyse mit Salzsäure wieder in Zucker zurückverwandelt werden kann. Diese Umwandelung scheint eine vollständige zu sein, wenn der Presssaft mit $^3/_1$-normaler Säure 3 Stunden erwärmt wird; andererseits erwies sich 7-stündiges Erhitzen mit $^1/_2$-normaler Säure in gewissen Fällen (vergl. z. B. die Versuche No. 192, 193, 196, 197 und 198) als nicht ausreichend zu vollständiger Zurückverwandelung der veränderten Substanz in Zucker.

Es scheint demnach, als ob die Differenz zwischen dem verbrauchten Zucker und der Summe von entstehendem Kohlendioxyd + Alkohol auf die Umwandelung eines Theiles des Zuckers in eine nicht reducirende, aber durch Hydrolyse mit Säuren wieder in reducirenden Zucker überführbare Substanz bedingt ist. Ob diese Substanz kohlehydratartiger Natur ist, konnte noch nicht ermittelt werden, da alle Versuche, sie zu isoliren, scheiterten. Ihre Bildung ist wahr-

scheinlich der Wirkung eines specifischen, von der Zymase verschiedenen Enzyms zurückzuführen, da das Phänomen sowohl in Presssäften von hoher fermentativer Kraft, wie auch in solchen (vergl. die Versuche No. 186—188 in Tabelle XV) von nur geringer Gährwirkung eintritt.

Tabelle XV.

Beziehungen zwischen dem verbrauchten Zucker und den entstehenden Producten.

(Diese Versuche No. 186, 199 und 200 wurden mit Toluol, die übrigen mit Thymol angesetzt; 25°.)

o.	Datum	Zeit in Stunden	Vol. des zugesetzten Wassers	Zugesetzte Glucose in g	Aus dem Kohlendioxyd berechneter Zucker in g	Bestimmung des Zuckers: direct		nach der Hydrolyse: mit $^1/_2$-*n*. Säure		mit $^3/_1$-*n*. Säure	
						Zucker verbraucht	Verlust an Zucker	Zucker verbraucht	Verlust an Zucker	Zucker verbraucht	Verlust an Zucker
8	6. XI. 02	84	0	2.2	1.31	1.42	0.11	1.40	0.09	—	—
8	18. IV. 02	72	0	2	0.07	0.43	0.36	0.03	-0.04	—	—
87	3. IV. 02	72	0	2.2	0.07	0.26	0.19	0.10	0.03	—	—
8	20. VI. 01	72	0	10	0.09	1.46	1.37	0.12	0.03	—	—
89	24. VI. 01	72	0	10	0.40	0.86	0.46	0.40	0	—	—
90	10. VII. 01	72	0	10	0.33	1.40	1.07	0.60	0.27	—	—
91	13. VI. 02	72	0	2.2	0.40	1.59	0.19	0.41	0.01	—	—
92	25. XI. 02	60	0	2	0.96	1.17	0.21	1.22	0.26	1.08	0.12
	»	60	0	2	0.98	1.19	0.21	1.28	0.30	1.11	0.13
93	»	60	1	4	1.00	1.41	0.41	1.53	0.53	0.94	-0.06
94	16. XII. 02	60	0	2	0.52	1.07	0.55	0.53	0.01	0.60	0.08
95	13. I. 03	64	0	2.2	0.50	0.88	0.38	0.54	0.04	0.45	-0.05
96	»	64	1	4.4	0.50	0.82	0.32	0.73	0.23	0.56	0.06
97	21. I. 03	64	0	2.2	0.59	0.77	0.18	0.72	0.13	0.60	0.01
98	»	64	1	4.4	0.53	0.81	0.28	0.63	0.10	0.48	-0.05
99	4. II. 03	64	0	2.2	0.69	0.92	0.23	—	—	0.71	0.02
00	»	64	0	2.2	0.70	0.95	0.25	—	—	0.70	0.00

Wird der Presssaft in der weiter oben beschriebenen Weise nach der Vergährung des Glykogens untersucht, so findet sich stets eine beträchtliche Zunahme der Reductionskraft des nicht hydrolysirten Presssaftes; diese ist wahrscheinlich auf die Wirkung des diastatischen Enzyms im Presssaft zurückzuführen. Die Menge des verbrauchten Zuckers stimmt, wie mit Hülfe der hydrolysirten Producte festgestellt werden konnte, genau so wie bei der Glucose, mit demjenigen Quantum überein, das sich aus den Gährungsproducten berechnen lässt (vergl. die Versuche No. 201, 203, 205, 207 und 210 in Tabelle XVI).

Tabelle XVI.

Beziehungen zwischen dem verbrauchten Zucker und den entstehenden Producten.

(25 ccm Presssaft, Thymol, 72 Stdn. bei 25°.)

No.	Datum	Zugefügtes Glykogen in g	Gewinn an Zucker	verbraucht. Zucker, aus dem Kohlendioxyd ber.	Zur Hydrolyse benutzte Säure			
					$^1/_2$-normal		$^3/_1$-normal	
					verbraucht. Zucker	Verlust an Zucker	verbraucht. Zucker	Verlust an Zucker
201	24. VI. 01	2	0.49	0.41	0.50	0.09	—	—
202	13. VI. 02	0	—	0.22	—	—	0.17	-0.05
203	do.	2	1.74	0.32	—	—	0.34	0.02
204	27. VI. 02	0	—	0.21	—	—	0.21	0
205	do.	0.8	0.33	0.36	—	—	0.38	0.02
206	18. IV. 02	0	—	0.06	0.06	0	—	—
207	do.	2	0.36	0.06	0.05	-0.01	—	—
208	25. XI. 02	0	—	0.21	0.28	0.07	0.26	0.05
209	13. I. 03	0	—	0.18	0.17	-0.01	0.20	0.02
210	do.	2	1.29	0.24	0.47	0.23	0.23	-0.01

Aehnlich liegen die Verhältnisse bei der Selbstgährung, doch lässt sich hier bei dem nicht hydrolysirten Presssaft keineswegs in allen Fällen eine Zunahme der Reductionswirkung feststellen.

XI. Zusammenfassung.

Als Resultat der im Voranstehenden beschriebenen Versuche ergiebt sich, dass der einzige, unverkennbar vorhandene Unterschied zwischen den von uns aus obergähriger Hefe und den von Buchner aus untergähriger Hefe dargestellten Presssäften in der geringeren Intensität der Gährung liegt, welche der Presssaft aus obergähriger Hefe in Glucoselösungen hervorruft; demzufolge gewinnt hier die Selbstgährung des Presssaftes eine relativ grössere Bedeutung. In jeder anderen Hinsicht scheinen sich die beiden Arten von Presssäften völlig gleich zu verhalten. Die chemische Veränderung, welche bei der Vergährung von Glucose eintritt, scheint eine wirkliche »alkoholische Gährung« zu sein, bei der sich annähernd gleiche Mengen Kohlendioxyd und Alkohol bilden.

Mit dieser dürfte eine andere Veränderung parallel gehen, bei welcher ein gewisser Betrag des Zuckers in nicht reducirende Stoffe übergeführt wird, die ihrerseits durch Hydrolyse mit Säuren wie-

derum in reducirenden Zucker zuruckverwandelt werden können. Die Natur dieser Substanzen, sowie der Vorgänge, denen sie ihre Entstehung verdanken, hat noch nicht aufgeklärt werden können.

Chemical and Water-Laboratory.

Lister Institute of Preventive Medicine, 30. Januar 1904.

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The Alcoholic Ferment of Yeast-Juice. By ARTHUR HARDEN and W. J. YOUNG.

(*From the Lister Institute of Preventive Medicine.*)

The alcoholic fermentation of glucose by yeast-juice is greatly increased by the addition of yeast-juice, which has been boiled and filtered, either when fresh or after undergoing autolysis, although this boiled liquid is itself incapable of setting up fermentation. Thus, the total fermentation produced by 25 c.c. of yeast-juice acting on 2·5 grms. of glucose is, on the average, doubled by the addition of an equal volume of the boiled juice and increases to a maximum when 3—5 volumes are added, after which it decreases.

The rapid loss of fermentative power, which is a characteristic property of yeast-juice, has been attributed to the destruction of the alcoholic ferment, or zymase, by the active proteolytic enzyme (endotryptase) which is also present. The increase caused by the addition of the boiled juice might therefore be due either to an actual increase in the activity of the alcoholic ferment, or to a decrease in the activity of the proteolytic ferment, or to both these effects. The results obtained by us point to the fact that the main effect of the addition is to increase the activity of the alcoholic ferment, whilst variation in the activity of the proteolytic ferment only plays a subordinate part.

The constituent of the boiled and filtered juice to which this effect is due has not yet been isolated, but it is characterised by the following properties, which are similar to those of the substances described by previous observers as co-ferments. It is not affected when its aqueous solution is boiled, it is dialysable, and it is precipitated by 75 % alcohol.

The proof that the alcoholic fermentation produced by yeast-juice depends on the presence of this substance and cannot take place in its absence has been brought by the use of Martin's gelatine filter. When yeast-juice is passed through such a filter and the residue which is left on the filter is dissolved in water, it is found that neither this residue nor the filtrate can by itself ferment sugar but that a mixture of these two produces a fermentation almost equal to that of the original juice.

The proteolytic enzyme on the other hand does not appear to possess a dialysable co-ferment, since the residue by itself readily undergoes autolysis in solution, and the extent of this is not materially altered by the addition of the filtrate.

Further experiments are in progress.

THE CONTRIBUTION OF EXPERIMENTS WITH SNAKE POISONS TO OUR KNOWLEDGE OF ANTI-TOXIC IMMUNITY.

By C. J. MARTIN, M.B., D.Sc, F.R S

(Contribution to the Discussion of Immunity, British Medical Association, Oxford, 1904.—from the British Medical Journal, 1904, p. 574).

No apology is, I think, necessary on behalf of those of us who have worked at the subject of snake venoms on account of its intrinsic interest. The fact that these reptiles are endowed with a poison of such potency that in some cases 0.000025 g., or even one-tenth of this quantity, if introduced intravenously, will speedily destroy a rabbit of 1,000 grams weight, is in itself worthy of attention, both from a general biological and a pharmacological point of view. The toxicity of the most powerful venoms thus far exceeds that of the most potent alkaloidal poisons known.

I will not enter into the consideration of the chemical composition of venoms further than to point out that they consist for the most part of solutions of modified proteids, varying between 20 to 25 per cent. in strength, and that so far the attempts which have been made to separate the toxic principle from such proteids, as has been accomplished, for instance, by Jacoby[1] and Hausmann[2] for ricin and abrin, have been unsuccessful.

Accordingly, at the present time, one must regard such toxic principle as residing in some special grouping of a portion of the atoms in the complex venom-proteid molecule, although it is possible that this is a side chain, and may be ultimately capable of differentiation as a separate entity. Venoms are by no means simple solutions of one poisonous substance, but the more they have been studied the more complex have they been shown to be. The analysis of their physiological actions has proved them to be made up of a great many more constituents than would be imagined from their chemical examination.

Different venoms have been found to contain one or more of the following :

1. A powerful fibrin ferment. (Lamb, Martin.)

2. A neurotoxin with in many cases a special affinity for the cells of the respiratory centre. (Every investigator).

3. A neurotoxin with an affinity for nerve endings in muscle and those in the diaphragm in particular. (Brunton and Fayrer, Elliott.)

4. Various cytolysins, for example, for red blood cells, endothelium of vessels, leucocytes, nerve cells, and the cells of many other tissues. (These cytolysins are distinct for each cell, and are of the nature of amboceptors.—Flexner and Noguchi.)

5. An antibactericidal body. (Welch and Ewing.) (These are of the nature of anticomplements.—Flexner and Noguchi.)

6. An antifibrin-ferment. (Cunningham, Lamb.)

7. Agglutinin for red blood cells, etc. (Weir Mitchell and Reichert, Flexner and Noguchi.)

8. A proteolytic ferment. (Weir Mitchell, Flexner and Noguchi.)

9. A substance which causes systolic standstill of the isolated heart. (Elliott).

Nearly all venoms contain a neurotoxin, and the rapidly fatal action of some venoms is due either to this constituent causing respiratory paralysis, or to the action of the fibrin ferment producing intravascular clotting.

No venom yet studied is possessed of all the above actions, and the dissimilarity in the effects produced upon man and animals after poisoning with the venoms of different snakes is due to the proportions in which the different groups of toxic substances occur in the particular venom.

Some years ago, in a not unnatural striving after unity, I expressed the opinion that all these constituents with the same physiological action, which have been found in different venoms, might indeed be identical. My reasons for this view were: (1) the similarity of the action of the neurotoxin, say, of cobra and *Notechis scutatus* (an Australian snake), or of the fibrin ferment in the poison of *Notechis pseudechis*, and *Echis carinatus*. (2) the repeated assertion of Calmette that the antiserum against one species of venom was equally powerful against others. Recent experiments by Cunningham, Lamb, Tidswell, Flexner and Noguchi, Kyes, Rogers, and myself have, however, led me to the conclusion that many of these constituents from different venoms, even when incapable of being differentiated by their physiological action, can be so differentiated by the inability of antiserum prepared against one venom to combine with the toxin in another venom; or, expressed in terms of Ehrlich's interpretation, that, although the toxophoric group may be similar, the haptopher is generally dissimilar.

It is not, however, on account of its intrinsic interest that I wish to draw your attention to the subject of snake venoms to-day, but because, owing to the analogy which has been found to exist between venoms and the toxins elaborated by some micro-organisms, the results of experiments with snake poison have taken a not unimportant part in the development of our knowledge of immunity.

The full strength of the analogy was demonstrated in 1894, when almost simultaneously Calmette and Phisalix and Bertrand published the fact that by continued treatment of an animal with injections of venom they had been able to produce an antitoxic serum. This observation was confirmed by Fraser in 1896. Since then, principally owing to the energy of Calmette, antivenene has taken its place as a remedy for poisoning by quite a number of snakes.

There is, however, an important point in connection with the therapeutic value of antivenenes which is in a very unsatisfactory condition—namely, how far are they specific?

Calmette has frequently emphatically stated that they are not specific, but this has not been found to be the case by Cunningham, Kanthack, Lamb, Tidswell, Rogers, Flexner and Noguchi, and myself, working with a variety of venoms and antivenenes prepared from a single kind of venom.

In a recent paper Calmette[3] goes so far as to admit that there are two poisonous constituents in some venoms, namely, a neurotoxin and a haemorrhagin (Flexner), and that the antitoxic serum prepared by injecting cobra venom is, as shown by Flexner, without action upon this haemorrhagin. He states further that one can easily obtain polyvalent antivenenes by vaccinating animals simultaneously with a strongly neurotoxic poison, such as cobra venom, and with a venom rich in haemorrhagin, such as that of the vipers *Daboia russellii* or *lachesis.*

In support of this conclusion he says that the "serums obtained by vaccinating horses exclusively with the venom of the cobra is distinctly (*nettement*) antitoxic with regard to all the neurotoxins of other venoms." He gives no experimental data, and I feel in some doubt as to the precise meaning of this statement. In the same communication he makes the further statement that the serum prepared in Sydney by Tidswell "destroys very well *in vitro* the venoms of the two Australian snakes *Hoplocephalus* and *Pseudechis.*"

Now, this is the very opposite of the facts described by Tidswell, and which I have been able to confirm. It was with the object of determining this question of specificity that Tidswell immunized a horse with the poison of *Hoplocephalus curtus*. He ultimately procured a serum 0.4 c.cm. of which neutralized 10 M.L.D. for 1,000 g. rabbit of the same poison, but 4 c.cm.(10 times the quantity) produced no appreciable effect upon 10 M.L.D. of the venoms of three other Australian snakes, which were all colubrines, and two of them belonging to genera closely-allied to *Hoplocephalus*. The physiological action of the poisons of these two—*Pseudechis* and *Dimenia*—is absolutely similar to that of *Hoplocephalus*.

Lamb[4] has also made a long series of careful experiments bearing upon the question of specificity. He used a serum which he procured by immunizing horses with pure cobra venom, and Tidswell's serum against *Notechis* (*Hoplocephalus*) poison, and tested these against 10 M.L.D. of the following venoms: *Naja tripudians*, *Naja bungarus* (king cobra), *Bungarus coeruleus*, *Bungarus fasciatus*, *Enhydrina valakadieri*, *Daboia russellii*, *Hoplocephalus curtus*, *Echis carinata*, *Trimeresurus gramineus*, *Crotalus adamantus*.

Using quantities of these sera from 4 to 10 times those necessary to neutralize 10 M.L.D. of the particular poison by injections of which the serum has been produced he obtained no delay in time of death; 15 times this quantity of anti-cobra serum caused delay in time of death when mixed with the venom of king cobra, and was sufficient to prevent the death of the animal when mixed with the venom of *Enhydrina valakadieri*.

Rogers's[5] results with Calmette's antivenene indicate a less rigid specificity for serums, but it must be remembered that this antivenene is made by vaccinating with a mixture of venoms. It appears that in all cases the serums are not absolutely specific, but neutralization may sometimes be

accomplished by *greatly* increased quantities of a heterogeneous serum.

The question of specificity can conveniently be studied in regard to haemolysin, which is present in almost all venoms; and this has been done by Stephens, and quite recently in a very careful manner by Lamb.

Stephens,[6] using an antivenene of Calmette's prepared by injecting horses with the venoms of *Naja haje* 80 per cent., *Bothrops lanceolatus* 10 per cent., *Crotalus* 10 per cent., concludes that the haemolysin of a variety of venoms can be neutralized by an antivenene made by vaccinating horses with snake poisons other than those used in the experiments, but that its antihaemolytic power is by no means equal against the different venoms.

Lamb[7] has tested the antihaemolytic power of the serum of a horse immunized with pure cobra poison against ten different venoms, and finds that, whereas with a M.H.D. of cobra venom haemolysis is prevented by 0.03 c.cm. of serum, thirty times this quantity of serum had *no* effect on the M.H.D. of *Bungarus fasciatus*, *Hoplocephalus curtus*, *Vipera russellii*, *Echis carinata*, *Trimeresurus gramineus* or *Crotalus adamantus*, and that the M.H.D. of *Bungarus coeruleus* required twenty times this quantity of serum. With *Naja bungarus* and *Enhydrina valakadieri* nearly complete haemolysis took place with 1 c.cm. of serum, that is, 30 times the quantity adequate to prevent solution of the corpuscles by a M.H.D. of cobra venom.

These experiments are, in some details, in apparent contradiction to those of Stephens, but it must be remembered that Lamb refers to an antivenene produced by the injection of a single venom and Stephens by a mixture.

These experiments show that the haemolysins are largely specific, and that for the purpose of serum therapeutics one must not assume identity from similarity of physiological

action. Megatheriolysin and tetanolysin both dissolve corpuscles.

This subject of specificity is too large a one to enter into more fully at the present time. The differences are as to fact, and can only be determined by evidence adduced. In fairness to the other observers quoted I must say, however, that I have never been able to discover in the publications of Calmette the account of any experiment giving quantitative data which has convinced me of the accuracy of his contentions. Moreover, when the haptophor groups of the haemolysins in two different venoms happen to be the same, it by no means follows that the haptophors of the neurotoxins in the two venoms are identical.

From the evidence available I am of opinion that, notwithstanding identity in physiological action in the constituents of different venoms, the antibodies produced by their injection into animals are to a high degree specific.[8] Exceptions for the individual constituents do undoubtedly occur, as shown for haemolysins by Stephens, and Flexner and Noguchi who found that the antibody produced by the injection of *Crotalus* serum was effective against the haemolysins of all venoms they experimented with.

For researches of theoretical importance in connexion with the relationship of toxins and antitoxins, venoms possess an advantage over bacterial toxins owing to the fact that in a chemically dry condition they are perfectly stable. They are, moreover, fairly stable in watery solution if kept cold, and in most cases can be heated to 70° C. or even boiled without being destroyed. Further, most venoms contain a haemolysin which permits of experiments being conducted in test tubes instead of with animals, thereby greatly increasing the accuracy in all quantitative determinations, both by doing away with an insensitive end point (namely, the death of a guinea-pig) and permitting of a much larger number of determinations being made than would be practicable in animal experiments.

The nature of the antagonism between toxins and antitoxins was the subject of controversy during the first few years subsequent to the discovery by Behring of the principle of serum therapeutics.

Behring, Kitasato and Ehrlich were of opinion that the antagonism was of chemical nature and that the antitoxin neutralized the toxin much as an acid is neutralized by an alkali. In 1894, Ehrlich, Kossel, and Wassermann[9] made the important observation that the neutralization occurred according to the simple law of proportion (law of multiples) which indicated a simple chemical relationship between the two. Buchner[10] found this unconvincing and took the contrary view that the toxin was not directly destroyed by the antitoxin, but that the latter operated in some way upon the cells of the animal so as to render them insusceptible to the poison. This view was strongly supported by Roux.

In support of his views Buchner stated that a mixture of toxin and antitoxin which failed to kill mice was fatal to guinea-pigs, and quoted a statement of Behring's that an animal which in process of immunization against tetanus had become hypersensitive to the poison, nevertheless afforded an immune serum of high degree.

At the Eighth International Congress of Hygiene and Demography, 1894, as the strongest evidence in support of his view, Roux[11] communicated some results of Calmette's with snake venom and antivenom. Calmette used cobra poison and his newly-discovered antivenene. The former is unaltered by heating to 68° C. for ten minutes, but the antivenene is destroyed by this treatment. Mixtures of cobra poison and antivenene were allowed to remain together ten minutes and injected into rabbits. The rabbits showed no symptoms. Similar mixtures heated to 68° for ten minutes after mixing killed rabbits of the same weight in a few hours. From his experiments Calmette concluded that the toxin of snake venom does not interact with its antitoxin *in vitro* but only *in corpore,* and that the neutralization of

toxin by antitoxin is not to be accounted for by a chemical operation between the two. Subsequently somewhat analogous experiments were made by Wassermann[12] with pyocyaneus toxin and antitoxin, and by Marenghi[13] with diphtheria, which yielded similar results to those of Calmette.

In 1896 a highly important contribution to the immunity question was made by Fraser.[14] This observer used cobra venom and an antivenene which he obtained by immunizing rabbits against this poison. Having confirmed the fundamental results of Calmette, and having shown that after the first M.L.D. the law of multiples discovered by Ehrlich, Kossel, and Wassermann was valid for venom and antivenene, he proceeded to investigate the influence upon the result of varying the conditions under which both the venom and antivenene were administered. He made four series of experiments :—

(1) The venom and serum were mixed together prior to injection.

(2) The venom and serum were simultaneously injected under the skin of opposite sides of the body.

(3) The serum was injected some time previously to the venom.

(4) The venom was injected previously to the serum.

In the first series 0.25 c.cm. protected a rabbit against 1½ lethal doses. In the second series it required 3.5 c.cm. (eight times as much) to protect against 1½ lethal doses. In the third series 2.7 c.cm. were required, and in the fourth series 3.2 c.cm. were required.

Fraser pointed out that the great difference between the dose of serum required when the two substances, although simultaneously administered, are in the one case mixed together before injection and in the other not so mixed, suggests most forcibly that the antidotism is chemical and not physiological. Another very important fact observed by Fraser was that 1.3 c.cm. of antivenene neutralized 5 M.L.D. if allowed to remain in contact twenty minutes, but failed to

do so when intervals of five or ten minutes only elapsed between mixing and injecting. As pointed out by this observer, the "resistance of tissues" theory of Buchner, Roux, and Calmette is quite incapable of explaining these results; whereas they are entirely compatible with a chemical interpretation of the reaction between toxins and antitoxins.

Nothwithstanding the work of Fraser, the experimental evidence against the simple chemical interpretation advocated by Behring and Ehrlich must have seemed so convincing that in the opening paragraph of the latter's classical paper on ricin toxin and antitoxin[15] the following sentence is to be found: "As the original interpretation of a destruction of the poison by the antibody has been proved untenable, since it is admitted that in a physiologically neutral mixture both components are present as such, two opposing views present themselves:—

(1) That in the tissue fluids toxin and antitoxin do combine to form a harmless mixture.

(2) Antitoxin has no action upon toxin, but operates in some way upon the cells so as to immunize them. (Roux and Buchner.)"

In this paper Ehrlich showed that the agglutination *in vitro* of blood corpuscles by ricin could be prevented by antiricin just as the poisoning of an animal could be prevented by the injection of serum. A similar fact regarding an action *in vitro* of cobra venom had, however, been discovered a little earlier by Kanthack. Cobra venom when added to recently-shed blood inhibits coagulation (Cunningham). Kanthack demonstrated to the Physiological Society in October, 1896, that this action of cobra poison was destroyed by antivenene, but not by any other serum. This observation of Kanthack's was unpublished.[16]

A further important contribution to the subject of antitoxin immunity, in which the evidence was derived from experiments with snake poison, was made in 1898 by Stevens

and Myers.[17] This research consisted of experiments with the haemolysin of cobra poison and its neutralization by antivenene. The experiments were similar to those of Ehrlich with ricin, and the authors succeeded in proving that the antivenene neutralizes the haemolysin of cobra poison *in vitro* and that the law of multiples applied in this case also.

Simultaneously with the above researches (1897-8) the problem was attacked by Cherry and myself.[18] We used both diphtheria toxin and the neurotoxin of a snake venom in our experiments. It was conceivable that, even if toxin and antitoxin did chemically combine, as seemed to necessarily follow from many of the facts recorded above, such combination might possibly be dissociated by a temperature of 68° C., and so explain the results of Calmette, Wassermann, and Marenghi.

To decide this point some method of separating toxin and antitoxin, when existing side by side, which could not possibly cause a loose chemical compound to dissociate, was wanted.

A few years previously[19] one of us had described a method of separating colloids from substances of smaller molecular size by filtering, under pressure, through a porcelain filter, the pores of which were plugged with gelatine.

We knew that both diphtheria toxin and the neurotoxin of the venom we were using traversed such a filter but that the diphtheria antitoxin, as shown by Brodie, did not.[20]

Here, then, was a tool which was capable by simple physical means of effecting a separation of the two constituents if existing side by side in solution.

We mixed solutions of diphtheria toxin and snake venom with slight excess of their respective antitoxins, allowed them to stand half an hour, and then filtered through gelatine. Quantities of the filtrate corresponding to 30 to 50 lethal doses of the original solution were injected into guinea-pigs without the slightest effect.

If the toxins had remained unaffected beside their respec-

tive antitoxins, there was nothing to prevent them passing through the filter in virtue of their smaller molecular size, for with a mixture of toxin and normal serum the filtrate was about three-quarters as toxic as the original solution. As, however, they did not pass the gelatine, we concluded that they had entered into some sort of chemical relationship with the relatively large molecules of the antitoxins during their sojourn together prior to the filtration. We then repeated the experiment of Calmette. We took mixtures containing several lethal doses of venom and sufficient antivenene, so that after standing together for thirty minutes prior to injection into a rabbit, no symptoms were produced. Some of these mixtures we heated to 68° C. for ten minutes before injecting. This made no difference to the effect.

These results, while they were in entire agreement with the filtration experiments, seemed diametrically opposed to those of Calmette. As the experiments were so simple as hardly to leave any possibility of experimental error, we turned our attention to any differences in the conditions which obtained in the experiments of Calmette and our own. The only obvious difference was in the time the venom and antivenene had been in contact before the heating. By this time we had become impressed with the necessity of a chemical interpretation of the phenomena, in which case time, temperature, and proportion of active masses would probably exert an important influence upon the transaction.

The toxin and antitoxin of this venom are both of great molecular size and complexity. *A priori* one would expect the velocity coefficient of any reaction between such complex molecules to be a high one, and, in addition, from their great molecular weight, the solution will in proportion to the mass dissolved contain relatively few molecules; so that it is not surprising that any chemical operation in which they are concerned should, as first pointed out by Fraser, occupy a very appreciable time. The experiments, indeed, showed this very clearly.

Proportion of Toxin to Antitoxin.		Control Venom Only.	Time Allowed for Interaction of Toxin and Antitoxin, Temp. 20° to 23° C					
Antitoxin	Toxin.		2 mins	5 mins.	10 mins.	15 mins.	30 mins.	Injected unheated 8 mins.
c.cm.	2 fatal doses	Died 15 hours	Lived (very ill for 2 days)	Lived (ill 1 day)	Lived (no symptoms)	Lived (no symptoms)	Lived (no symptoms)	Lived (no symptoms)
c.cm.	3 fatal doses	Died 12 hours	Died 20 hours	Died 28 hours	Lived (ill 2 days)	Lived (ill 1 day)	Lived (no symptoms)	Lived (no symptoms)
c.cm.	4 fatal doses	Died 9 hours	Died 13 hours	Died 15 hours	Died 23 hours.	Lived (very ill for 2 days.)	Lived (no symptoms)	Lived (no symptoms)

The value of the factors time and influence of proportion of active masses, will be seen on reference to the table above, which represents the results of twenty-one experiments. On reading along any horizontal line, will be seen the influence upon the result of the time during which the toxin and antitoxin were allowed to operate upon each other, with proportion of active masses constant. On reading any vertical line the influence of varying proportions of active masses with time of operation constant is indicated. The thick line separates off the fatal results from those in which the rabbits lived. All other factors were kept constant. That is, we obtained the same results as Calmette, whenever we heated the mixture whilst it contained still one fatal dose of venom unacted upon by the antitoxin.

Our experiments, then, not only afforded further support to the chemical interpretation of toxin-antitoxin antagonism, but we were also fortunate enough to be able to show the invalidity of the most important evidence upon which the opposite contention was based.

The neutralization of venom by antitoxin was shown by Myers[21] to take place in a similar manner to that described by Ehrlich in his classical researches on the constitution of diphtheria poison. Myers also found that, as is the case with diphtheria, solutions of venom diminish in strength on keeping but still require the same quantity of antivenene for their complete neutralization. It is worthy of mention at the present time that in this paper Myers discusses the possibility of the toxin-antitoxin reaction being reversible, and points out that if such were the case it would be capable of explaining his partial neutralization results without the assumption of the multiple composition of the venom toxin he was dealing with (haemolysin), a view which has recently been strongly urged by Arrhenius and Madsen.

This assumption in itself is not, however, adequate to explain the diminution in toxicity with maintenance of antitoxin combining power which was found to gradually take place in solutions of venom.

Flexner and Noguchi[22] have made a number of very interesting experiments bearing upon toxin-antitoxin relationship by working with the venoms of American snakes (crotalus, copperhead, moccasin), and also with the blood serum of these reptiles which is itself poisonous. Some of their results I have already referred to. They succeeded[23] by means of experiments analogous to those of Wassermann and Takaki[24] in neutralizing the neurotoxin of copperhead venom by means of emulsion of brain without appreciably diminishing the haemolysin in this venom. Emulsions of other organs did not possess this effect. They found that the haemolysin and neurotoxin of their venoms in solution readily formed toxoids, but that haemorrhagin did not; they were, however, able to produce toxoid formation in this constituent by treatment with weak HCl, and subsequently[25] succeeded by immunizing animals against this haemorrhagin by means of toxoid injections. This is not only of theoretical interest but of practical importance, for it is only by

this method that Noguchi[26] has succeeded in producing a serum of value against rattlesnake, owing to the impracticability of immunizing an animal by repeated injections of the unaltered venom on account of the terrible sloughs occasioned by each inoculation.

I feel that an account of the part played by snake-poison researches in the building-up of the doctrine of immunity would be incomplete without the mention of the valuable contributions which have been made by Preston Kyes to the knowledge of those less simple phenomena where the reaction is complicated by the necessity for the interaction of a third body, as in the case in haemolysis and bacteriolysis by foreign serums.

Flexner and Noguchi[27] showed that haemolysis by venom was accomplished only with the co-operation of a third body, which is present in normal serum. Venom haemolysis, therefore, became an exceedingly convenient field to study such phenomena.

Kyes[28] has taken advantage of this opportunity, and has obtained results of the greatest interest and importance, but the discussion of which would lead me into another chapter of the immunity question, which I must reserve for another occasion. I think, however, that I have shown that the results of researches with snake poison have taken no mean part in contributing the materials out of which the fertile imagination of Ehrlich has produced the doctrine of immunity.

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THE ADMINISTRATION OF ANTI-TOXIC SERA BY INTRAVENOUS INJECTION.

BY C. J. MARTIN, M.D., D.Sc., F.R.S.,
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(Contribution to the Discussion on Serum Therapeutics, British Medical Association, Oxford, 1904, from the "British Medical Journal," 1904, Part II., Page 901.)

I find myself in such entire agreement and so completely content with the admirable survey of the present position of serum-therapeutics which we have just listened to from Dr. Goodall, that I cannot even pretend to discuss it. Under these circumstances I feel that the best thing I can do is to take this opportunity of laying before you some experiments which have an important bearing upon the practical application of those serums the real value of which is undisputed. These experiments show, to my mind, that under some circumstances the therapeutic value of antitoxic serums is very greatly enhanced by intravenous administration.

As long ago as 1896 Fraser[1] pointed out, in connection with his experiments upon antivenene, the great discrepancy between the quantity of serum required to save an animal from $1\frac{1}{2}$ lethal doses of cobra venom, according to whether the two were previously mixed together in a glass, or were simultaneously but separately injected under the skin.

During 1897 I repeated these observations of Fraser's with another snake venom and antivenene, and carried out at the same time a further series of experiments in which the

same venom solution was introduced subcutaneously and the serum intravenously.[2] As the serum at my disposal was a weak one and I had only a small quantity of it, I was obliged to work with only two fatal doses of venom. The animals used were rabbits. Briefly stated, the results of the experiments were the following:

I.—*Venom and Serum Mixed together Fifteen Minutes before Injection.*

No. of Fatal Doses.	Amount of Serum.	Result.
2	1.5 c.cm.	Died.
2	2.0 c.cm	Died.
2	2.2 c cm.	Lived.
2	2.4 c.cm.	Lived

II.—*Venom Injected Subcutaneously and Immediately afterwards Serum Introduced into a Vein.*

No. of Fatal Doses.	Amount of Serum.	Result.
2	1.5 c cm.	Died.
2	2.0 c cm.	Died.
2	2 2 c.cm.	Lived.
2	2.4 c.cm.	Lived.

III.—*Venom and Serum Injected Simultaneously but Separately under the Skin.*

No. of Fatal Doses.	Amount of Serum.	Result.
2	10 c.cm.	Died.
2	20 c.cm.	Died.
2	25 c.cm.	Died.

That the proportion of toxin to antitoxin necessary for neutralization should be approximately the same, whether they be (1) mixed in a glass or (2) the antitoxin be injected into the blood stream and the toxin subcutaneously, might be expected, as the nature of the antagonism between them is chemical, for if antivenene be introduced into the blood stream the antitoxin is there ready to neutralize the toxin as it is absorbed.

The much higher proportion of antitoxin to toxin required when they are separately introduced under the skin necessitates the inference that antitoxin is, relatively to the venom, slowly absorbed from the subcutaneous spaces. Our chemical knowledge of this poison in hoplocephalus venom and of the active principle in antivenene, together with what is known

of the physiological mechanism of absorption, is quite in accordance with the view that this antitoxin is only capable of slowly penetrating the capillary wall, whereas the venom passes through fairly rapidly. The constituent of the venom which was used in the above experiments is an albumose. It dialyzes slowly, can be filtered through a film of gelatine under pressure, although it does not pass through so readily as water or bodies of simpler molecular constitution.[3] It is rapidly absorbed by the blood vessels. An animal can be killed by subcutaneous injection of a large dose in a few minutes, and the result is not retarded by previous ligature of the lymphatics from the limb and the thoracic duct.[4]

On the other hand, Brodie[5] filtered antitoxic serum of diphtheria through gelatine, and found that the active properties of the serum remained with the proteids on the outside of the filter. Cherry and I confirmed this result with diphtheria antitoxin, and found the same for antivenene.[6] Both these antitoxins are bodies of great molecular size comparable with proteids and are, indeed, in probability merely serum proteids with some slight modification of their molecular architecture. The walls of the capillaries of the limbs are membranes possessed of permeabilities approximating to those of a film of gelatine, for Starling showed they were relatively, although not absolutely, impermeable to proteids.[7] If molecular size is the obstacle to proteid absorption from subcutaneous spaces, the same would apply to antitoxins.

Calmette has made the statement that antivenene is more rapidly absorbed than venom. He does not adduce any experimental proof for such a statement, and I cannot see that these results can bear any other interpretation than that the poison with which I was working was absorbed ten to twenty times as rapidly as the active principle in antivenomous serum.

The same fact cannot be so well demonstrated for diphtheria toxin owing to the latent period which always

elapses before the onset of symptoms after the injection of diphtheria toxin, but Behring has estimated that eight hours are saved by the introduction of diphtheria antitoxin intravenously.

From the analogy between the toxin of diphtheria and this constituent of the venom as regards the order of magnitude of their molecules, I felt justified in recommending my clinical friends in Australia to employ intravenous injections of antitoxin in all bad cases of diphtheria.[8] This has been the routine practice in both the General Hospital and the Children's Hospital in Melbourne during the last six years, and the results have been most encouraging, and in no case were the injections followed by any untoward results.

Intravenous injection of curative serum was employed by Calmette and Salimbeni[9] in the outbreak of plague at Oporto in May, 1899. From the results of their experience these authors strongly recommend this method of treatment, and Cairns[10] also formed a favourable opinion of intravenous injections in the small outbreak at Glasgow in 1901.

In 1902 Cairns[11] published an account of twenty severe cases of diphtheria, most of them suffering from pulmonary complications, in which he injected from 20,000 to 35,000 units into a vein. He was more than satisfied with the results; indeed he says, "The almost immediate improvement after intravenous injection of serum in such cases (pulmonary) is as striking in its results as it is difficult of explanation."

The experience in Melbourne, amounting to some hundreds of cases, has been equally gratifying, so that I would suggest that if at any time the physician desires to obtain the action of antitoxic serum in a hurry, he should put it into a vein.

There is no difficulty in performing the injection if the veins of the arm are distended by means of a ligature as in the operation of venesection. The skin over a prominent vein is cleansed and the needle passed directly into the vein.

The fact that it is in the vein can be ascertained by its mobility and by the escape of blood through the needle. Being satisfied that the needle is inserted into the vein, the barrel of the syringe is attached and the serum slowly injected.

As many antitoxic sera contain 0·2 per cent. of phenol, one naturally enquires whether the amount of carbolic acid contained may not be dangerous when directly introduced into the circulation.

The toxic dose of phenol taken by mouth is given as 10 grams. by Kobert and 10-20 grams. by Löw. Kunkel, however, considers these figures too high.

There are no observations recorded of the effect of phenol introduced into a vein on man. The nearest approach to the required information in toxicological literature is an observation by Nieden,[12] in which 3 grams. (about 0·04 per kilo.) of phenol introduced into the pleural cavity gave rise to severe toxic symptoms, which were recovered from.

In order to ascertain to what extent the carbolic is a source of danger in intravenous injections of serum, Dr. Cathcart has kindly made for me some experiments to determine the toxic and fatal dose of phenol by intravenous injection for animals, and has come to the following conclusions, that

(1) 0·025 gram. per kilo. produced no obvious ill effects.

(2) 0·05 gram. per kilo. produced transitory twitching, but the animal appeared quite well one hour later and suffered from no ill effects afterwards.

(3) 0·1 gram. per kilo. is about the fatal dose.

From Nieden's single observation, man would appear to be about twice as sensitive as animals weight for weight. One may, I think, conclude that it would be wiser not to exceed the limit of 0·01 gram. per kilo. at one injection. This imposes a limit of 350 cc. of serum, containing 0·2 per cent. carbolic, for a man of 150 pounds weight, and 70 cc. of such serum for a child of 30 pounds. As 10,000 units of

diphtheria antitoxin are contained in 25 cc. of the sera commonly employed in England one is well within the margin of safety even for children.

NOTE.—In a paper communicated to the Section for Tropical Diseases (BRITISH MEDICAL JOURNAL, September 17th, 1904, p. 670), Captain Leonard Rogers, I.M S., has repeated these experiments on the beneficial effect of administering antivenene intravenously with identical results.

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[*From the Proceedings of the Physiological Society*, Feb. 20, 1904.]

On the percentage of Fat in different types of Muscle.

By J. B. Leathes.

In the following table the results of estimations of the amount of insoluble fatty acids present as fats, soaps, etc. in the different types of muscle tissue in the rabbit and cat are given. The figures give the percentage of the total solids formed by the higher fatty acids obtained as follows. The muscles of two or three animals were taken for each set of estimations in rabbits; in the cat the estimations are for the muscles of one and the same animal. The muscles were carefully freed from all visible connective tissue, tendon, etc., minced, dried, and powdered finely. The powder was then dried *in vacuo* at 100° C. for two days, which is sufficient to give practically constant weight. The dry powders were extracted twice according to Rosenfeld's method and the combined alcohol and chloroform extracts saponified with alcoholic potash. The soaps dissolved in a large volume of water were precipitated with sulphuric acid and heated till a sharp separation had taken place. The precipitated fatty acids were filtered off hot, washed with large quantities of hot water, and then dried *in vacuo* on the filter. The filter and precipitates were then extracted with petroleum ether in Soxhlet's apparatus, and the extracts filtered, dried, and weighed.

The figures show that the red muscles of the rabbit, like the diaphragm and heart, contain a very considerably greater store of fat than the white muscles and than the mixed muscles of the cat.

	Rabbit			Cat
	I	II	III	
[1]Soleus	7·19	7·97	15·19	4·23
Semi-membranosus	2·94	3·01	4·62	3·99
Diaphragm	—	6·60	11·62	8·09
Psoas	—	—	3·76	—
Adductors of thigh	—	—	4·62	—
Heart	—	—	12·33	12·89
Intestine	—	—	—	4·09

[1] Together with the soleus other conspicuously red muscles were taken, the semitendinosus and pectineus, to make up a sufficient weight of muscle for an estimation.

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[*From the Proceedings of the Physiological Society*, Feb. 20, 1904.]

On the Synthesis of higher fatty acids in the Liver. By O. HILDESHEIM and J. B. LEATHES.

In a former communication to this society we described six experiments, in which the liver of rabbits, reduced to pulp and suspended in saline solutions or diluted blood, was found to contain from 10 to 40 % more fat after incubation in a current of moist air for one to three days than it did when fresh. And in one of these experiments flasks to which glycogen was added gave double the increase found in flasks to which no glycogen was added.

The estimations were made by Rosenfeld's method, in which the dried substance is boiled with alcohol, then extracted with chloroform, and the alcohol and chloroform residues taken up in ether and weighed. This extract is of course not pure fat, as is shown by the fact that after saponification it yields only 60 to 65 % or less of its weight of insoluble fatty acids instead of about 95 % as yielded by pure animal fat from the connective tissues. But since the percentage of insoluble fatty acids was no higher in the extracts of the fresh liver than it was in samples that had been incubated and in which the extract had increased, there must have been a similar increase in the higher fatty acids.

Since then ten more experiments have been carried out by one of us (Pig's liver 3, Rabbit's liver 7) in which the higher fatty acids have been estimated after saponification of the extracts obtained as Rosenfeld obtains them.

In six of these ten experiments the higher fatty acids were increased during incubation by amounts varying from 15 to 33 %; in the four others there was either no change or an increase amounting only to 4 or 5 %, which may be neglected; but in no case was a diminution observed.

In three experiments glycogen was added to some of the flasks of liver pulp before incubation. These flasks showed a greater increase than the control flasks to which no glycogen had been added;—in one case 18 % gain as compared with 13 %, in another 21 % against 9 %.

In one experiment in which glycogen was added there was no increase either in the flasks with added glycogen or in the controls with none.

Immersion of the flasks before incubation into boiling water prevents the increase taking place, a short immersion partly, a longer one completely.

Putrefaction was prevented by the addition of antiseptics, but absolute sterility has not yet been attained. The organisms present were however not responsible for the increase of the fatty acids. For broth cultures of the organisms present in flasks in which the increase had amounted to 20 % caused no change in the amount of higher fatty acids in samples of boiled liver on incubation, as compared with other boiled samples to which previously boiled cultures from the same flasks were added.

That the increase could be accounted for by the liberation of fat during autolysis from combinations that occur in the fresh liver and in which it escapes extraction is improbable, for Rosenfeld showed that his method of extraction gave as much or more extract than could be obtained by the Pflüger-Dormeyer method in which peptic digestion precedes extraction, and we have found that the insoluble fatty acids obtained by the method we have followed are not less than those obtainable from the liver when heated with strong aqueous potash till dissolved, and then the saponification completed in the presence of alcohol, as in Liebermann's method of fat estimation.

Further details of these and other experiments, and as to the effect of the addition of various substances on this reaction, will be published shortly.

CLI.—*The Fractional Hydrolysis of Amygdalinic Acid. iso*Amygdalin

By Henry Drysdale Dakin.

When two optical antipodes separately combine with a structurally symmetrical substance, they do so with equal velocity, but if they combine with a substance containing an asymmetric group, the speed with which combination occurs may be different in the two cases, and, conversely, the rates of decomposition of the products formed by such reaction may also be unequal.*

* The combination of dextro- and lævo-isomerides with a substance containing an asymmetric group does not, however, necessarily take place with unequal speed.

Very few reactions have been described which illustrate these points, the best known being those studied by Marckwald and McKenzie (*Ber.*, 1899, **32**, 2130; Trans., 1904, **85**, 379), and by Walden (*Ber.*, 1899, **32**, 2703). These investigators have shown that the rates of esterification of optically isomeric acids with active alcohols are in many cases different, and also that the rates of hydrolysis of the partially racemic isomeric esters produced by such reactions may be unequal. The present paper gives an account of a reaction which is an example of the complete resolution of a partially racemic glucoside into active compounds without the help of additional optically active substances.

Amygdalinic acid (Liebig and Wöhler, *Annalen*, 1837, **22**, 11) is usually assumed to be a maltoside, and the formula

$$C_{12}H_{21}O_{10}\cdot O\cdot CHPh\cdot CO_2H,$$

representing it as the maltoside of mandelic acid, is usually accepted. Amygdalin, the parent substance of amygdalinic acid, gives *l*-mandelic acid on hydrolysis with hydrochloric acid, but amygdalinic acid itself, contrary to expectation, has been shown by J. W. Walker (Trans., 1903, **83**, 472) to give inactive mandelic acid. Amygdalinic acid is therefore to be regarded as the maltoside of inactive mandelic acid, or, as is more convenient to assume for the present, a mixture of *d*-mandelic acid maltoside and *l*-mandelic acid maltoside.

It is clear that these two forms, of which amygdalinic acid is assumed to be composed, are not optical antipodes, and, as might be expected, they undergo chemical change at different rates. Although, as previously stated, amygdalinic acid gives inactive mandelic acid on complete hydrolysis, it was found on fractional hydrolysis that the mandelic acid liberated in the early stages was strongly dextrorotatory, whilst the acid liberated towards the close of the reaction was lævorotatory. By suitable methods it was found possible to isolate the pure *d*- and *l*-mandelic acids from the products of hydrolysis, and thus obtain both isomerides from amygdalin by the successive action of barium hydroxide and hydrochloric acid, although amygdalin itself, on direct hydrolysis with acids, yields the lævo-acid almost exclusively.

Preparation and Hydrolysis of Amygdalinic Acid.

A large number of experiments on the fractional hydrolysis of amygdalinic acid were made which differed slightly in detail, but the following description of an actual experiment may be taken as being fairly typical.

For example, no difference in the rate of change has been observed in the case of reactions which depend simply on the relative affinities of the reacting substances, as in salt-formation between asymmetric acids and alkaloids (Marckwald and Chwolles, *Ber.*, 1898, **31**, 783).

Twenty-five grams of amygdalin were heated with a solution of 12·5 grams of crystallised barium hydroxide in 200 c.c. of water until the liberated ammonia was completely expelled. The excess of barium hydroxide was then removed by carbon dioxide and the solution of barium amygdalinate concentrated to 75 c.c. Twenty-five c.c. of hydrochloric acid (sp. gr. 1·16) were now added, the resulting liquid giving a rotation of $-12·96°$ in a 1 dcm. tube. Partial hydrolysis was effected by heating the solution on the water-bath until the rotation in a 1 dcm. tube had changed to $+2·67°$, this change being completed in about 20 minutes. Under these conditions, rather less than two-thirds of the amygdalinic acid was decomposed. After hydrolysis, the solution was rapidly cooled, and the liberated mandelic acid removed by means of ether in a continuous extraction apparatus; the acid thus obtained, which weighed 4·5 grams and had a specific rotation of $+17°$ ($c=5·942$; $\alpha=+1·02°$; $l=1$), corresponding with 11 per cent. of the dextro-acid, crystallised readily and was quite free from reducing sugar.

The aqueous solution containing the remainder of the unchanged amygdalinic acid was now heated on the water-bath for an hour in order to complete the hydrolysis; the mandelic acid (about 3 grams) was isolated by ether extraction as before, and gave in aqueous solution containing 5·168 grams in 100 c.c. a rotation of $-1·07°$ in a 1 dcm. tube, whence $[\alpha]_D-20·7°$, corresponding with about 13 per cent. of the lævo-acid.

It is thus clear that the mandelic acid first set free by the hydrolysis of amygdalinic acid is strongly dextrorotatory, and that the acid set free towards the close of the reaction is correspondingly lævorotatory. Numerous experiments were made, which completely confirmed Walker's statement (*loc. cit.*) that the mandelic acid set free on complete hydrolysis is optically inactive.

The more or less active mandelic acids obtained by a repetition of the foregoing experiment were combined, and the approximately pure active acids were isolated by fractionally crystallising both magnesium salts and the acids themselves.

Isolation of d-*Mandelic Acid.*—The mandelic acid formed during the earlier stages of the hydrolysis of amygdalinic acid was converted into the magnesium salt by warming its dilute aqueous solution with magnesium carbonate until the liquid was no longer acid to litmus. On filtering and concentrating, three main crops of crystals were obtained, of which the first, consisting almost entirely of inactive magnesium mandelate, was much the largest. The percentage of magnesium was checked in each crop as a precaution against possible admixture with other acids.

	1st Crop.	2nd Crop.	3rd Crop.
Percentage of Mg in salt dried at 100°, $C_{16}H_{14}O_6Mg$ requires Mg = 7·45.	7·5	7·7	7·7
Specific rotation in 3 per cent. HCl ...	nil	+29·3	+64·7

The second crop was recrystallised and the mother liquor worked up with the third fraction. The most soluble magnesium salt was finally decomposed with sulphuric acid and the mandelic acid recovered by means of ether. The ethereal extract was evaporated, the residue dissolved in water, filtered from a trace of oily impurity, and slowly concentrated on the water-bath. The mandelic acid was then crystallised repeatedly from water, when the active acid, being less soluble than the racemic acid, separated first. A small quantity of acid was finally obtained, which melted at 132° and had $[\alpha]_D^{16°}$ +158°. There can be no doubt that the substance was practically pure *d*-mandelic acid, which melts at 132·8° and has $[\alpha]_D^{20°}$ +156·6° (Lewkowitsch, *Ber.*, 1883, **16**, 1568).

Isolation of l-*Mandelic Acid.*—The mandelic acid set free during the later stages of the hydrolysis of amygdalinic acid was converted into the magnesium salt and fractionated in the manner indicated for the dextro-acid. Three main fractions were obtained, as before, which gave respectively Mg = 7·3, 7·6, and 7·6, and α_D in 3 per cent. hydrochloric acid −3·0°, −7·9°, and −69·6°.

On recrystallising and then recovering the mandelic acid from the most soluble fraction, a product was obtained which melted indefinitely between 115° and 125°. After three recrystallisations from water, the crystals melted at 131—132°, and when examined in aqueous solution had a rotation of −152°. These constants agree well with those given by Walden, namely, m. p. 131—132°, $[\alpha]_D$ −153° (*Zeit. physikal. Chem.*, 1895, **17**, 706). These numbers are a little lower than those given by Lewkowitsch (*loc. cit.*).

Although the fractional hydrolysis of amygdalinic acid furnishes a fairly easy method for the preparation of the active mandelic acids, the yield of pure substance is extremely small.

iso*Amygdalin.*

Amygdalin, when boiled with concentrated acids, yields *l*-mandelic acid together with glucose and ammonia, but when treated with alkalis in hot solution gives amygdalinic acid (Liebig and Wöhler, *Annalen*, 1837, **22**, 11). The latter acid, however, on acid hydrolysis furnishes racemic mandelic acid, and not the lævo-modification. J. W. Walker, who first recorded this fact (Trans., 1903, **83**, 472), also showed that the racemisation of the mandelic acid part of the glucoside molecule was not a result of the conversion of the nitrile

group into the carboxyl group, for racemisation was complete before this further reaction had taken place. Amygdalin, when dissolved in cold barium hydroxide solution without any evolution of ammonia taking place, was found to yield inactive mandelic acid on subsequent hydrolysis with mineral acids. This apparently catalytic racemisation by means of alkalis recalls the conversion of active hyoscyamine into atropine by means of bases, and of tartaric acid into racemic acid by means of the oxides of iron and aluminium (Will and Bredig, *Ber.*, 1888, **21**, 2777; Jungfleisch, *Compt. rend.*, 1877, **85**, 805). It is interesting to note that, in being racemised with exceptional ease, amygdalin as a cyanogen derivative, shows a close resemblance to the comparatively few known active halogen compounds (compare Walden, *Ber.*, 1898, **31**, 1416). Walker suggested that the first product of the action of alkalis on amygdalin was an isomeric "racemoid" amygdalin, but his attempts to isolate the substance resulted in the production of uncrystallisable gums. This substance has now been isolated in the course of an investigation on the action of enzymes on derivatives of amygdalin. The composition and reactions of the new compound, to which the name *iso*amygdalin is assigned, closely resemble those of the parent substance, as might be expected if the two compounds are optical isomerides.

*iso*Amygdalin is easily prepared by shaking up 5 grams of amygdalin with 25 c.c. of a cold barium hydroxide solution containing 1·25 grams of the crystalline hydroxide. After about 15 minutes, carbon dioxide is passed into the liquid until it is no longer alkaline to phenolphthalein, and the mixture is gently warmed on the water-bath for a few minutes; the precipitated barium carbonate is then removed and the filtrate concentrated to a clear colourless syrup, which, when vigorously stirred with ethyl acetate, slowly yields an almost solid mass of glistening, white plates and needles. These crystals are drained on porous earthenware, triturated with more ethyl acetate, and again drained; they retain the ethyl acetate very tenaciously.

For analysis, the substance was dried in a vacuum over phosphoric oxide at about 50°.

0·1605 gave 0·2859 CO_2 and 0·0913 H_2O. C = 48·7; H = 6·3.
0·1497 „ 0·0413 gram Nitrogen. N = 2·7.
$C_{20}H_{27}O_{11}N,2H_2O$ requires C = 48·8; H = 6·3; N = 2·8 per cent.

*iso*Amygdalin crystallises with 2 molecules of water, whereas ordinary amygdalin contains 3 molecules of water; this may be removed by heating the substance at a temperature slightly above 100°, but the product is somewhat discoloured, so that it is better to analyse the substance dried to constant weight at a lower tempera-

ture. The substance containing 2 molecules of water of crystallisation has no definite melting point, but at about 100° effervescence occurs, water is expelled, and a viscid, glassy mass is left. If, however, the substance is previously dried at 120°, it melts gradually on further heating between 125° and 140° to a slightly yellow, viscid liquid.

*iso*Amygdalin, as was to be expected from Walker's experiments, is lævorotatory. A solution containing 2·688 grams of the crystallised salt in 100 c.c. had a rotation $-1·28°$ in a 1 dcm. tube, whence $[\alpha]_D^{20°}$ $-47·6°$. The substance differs very considerably from amygdalin in being extremely soluble in water, readily soluble in ordinary alcohol, much less so in absolute alcohol, and almost insoluble in ethyl acetate. The substance is not easily recrystallised except by almost complete evaporation of the solvent. An aqueous solution does not reduce alkaline copper sulphate solution provided that prolonged boiling and a large excess of alkali are avoided. On adding strong sulphuric acid, the substance, like amygdalin, develops a crimson-red coloration, which disappears on dilution with water. This reaction is considered by Schiff (*Ber.*, 1899, **32**, 2699) to be characteristic of the group, $C_6H_5·CH(CN)·O-$.

*iso*Amygdalin, which has a bitter quinine-like taste, indistinguishable from that of ordinary amygdalin, yields ammonia and amygdalinic acid on warming with alkalis, whilst with acids it gives mandelic acid, *d*-glucose, and ammonia. Further details of the hydrolysis by means of acids will be given later.

A proof of the close relationship existing between *iso*amygdalin and amygdalin is the fact that they are both similarly attacked by the enzymes emulsin and maltase. A solution of emulsin was prepared according to Bourquelot's method (*Bull. Soc. Mycol. de France*, 1896, 11, [iii], 199) by macerating mature growths of *Aspergillus niger* with water. After some days, a good deal of autolysis was found to have taken place, and emulsin and other enzymes had dissolved. On adding a little of the liquid to solutions of either amygdalin or *iso*amygdalin and digesting for a short time, the odours of benzaldehyde and hydrocyanic acid were quite marked, and the solution reduced Fehling's solution to a much greater extent than did those from control experiments in which the enzyme had been previously destroyed by boiling. Qualitatively, the two isomerides behaved similarly in yielding the same final products, although it is improbable that the speed of the reaction is the same in both cases.

A solution of maltase was prepared by digesting for one day at 35° the emulsion obtained by grinding air-dried yeast with sand and water. Experiments were made in which portions of 5 c.c. of the filtered extract were digested with 0·5 gram of amygdalin or *iso*amygdalin,

control experiments being carried out simultaneously. It was found that after digestion both solutions had a much increased reducing action on Fehling's solution, and crystals indistinguishable from phenylglucosazone were prepared by suitable methods. It is, therefore, probable that the action of maltose on *iso*amygdalin is similar to that on amygdalin (E. Fischer, *Ber.*, 1895, **28**, 1509). No attempt was made to isolate the partially racemic mandelonitrile glucoside which, together with *d*-glucose, is probably formed by the action of the enzyme.

The action of acids on *iso*amygdalin is in some respects peculiar. The mandelic acid liberated on complete hydrolysis of the substance with hydrochloric acid was not completely inactive, as one would expect, but distinctly dextrorotatory. This result is the more surprising, since amygdalin itself gives *l*-mandelic acid almost exclusively. As an example, the following typical experiment may be described: one gram of *iso*amygdalin and 5 c.c. of hydrochloric acid (sp. gr. 1·12) were heated together for 30 minutes at 80°; the resulting solution was diluted, extracted with ether, and the mandelic acid recovered by evaporation of the solvent; the acid had $[\alpha]_D + 16°$ in aqueous solution. In other experiments differing only in detail, acids were obtained with rotations varying from $+80°$ to $+20°$.

That the rotations were really due to *d*-mandelic acid and not to traces of glucose was proved by showing that the optical activity persisted after crystallisation of the mandelic acid from a mixture of petroleum and ether.

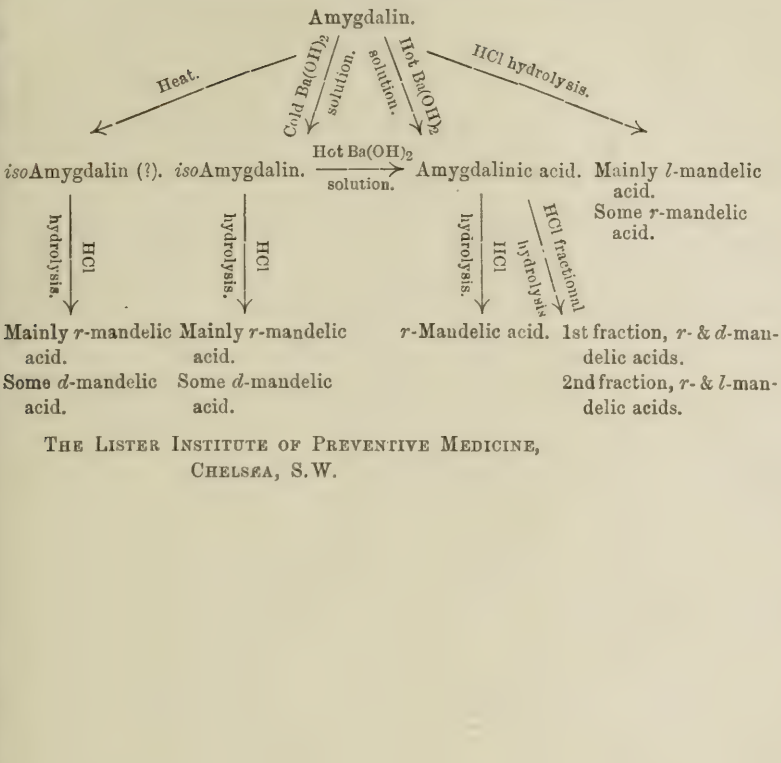
If cold alkalis act on amygdalin to produce a mixture of *i*-mandelonitrile maltoside and *d*-mandelonitrile maltoside, then the production of some *d*-mandelic acid in the hydrolysis of *iso*amygdalin would be readily explained, but such a reaction appears very improbable, although perhaps not impossible in the light of Walden's work on the interaction of bases and active halogen acids. A more probable explanation is that *iso*amygdalin should be considered to be entirely composed of *i*-mandelonitrile *d*-maltoside. This substance may exist as a mixture of the two forms, *d*-mandelonitrile maltoside and *l*-mandelonitrile maltoside. These two forms would not be optical antipodes, and hence would probably be hydrolysed at unequal rates. If now the dextro-compound be assumed to be hydrolysed more rapidly than the lævo-isomeride, at any point short of complete hydrolysis the mandelic acid set free would be dextrorotatory, whilst the unchanged glucoside would contain an excess of its lævo-component. Now it is clear, from what has been previously stated, that this lævo-component, which is ordinary amygdalin, is racemised with extraordinary ease as regards the mandelic acid portion of the molecule, and if it is assumed that this

excess of *l*-mandelonitrile maltoside is being both racemised and hydrolysed, the final result must obviously be the production of a certain amount of *d*-mandelic acid.

This explanation is supported by the fact that when ordinary amygdalin is hydrolysed by acids and the liberated mandelic acid quantitatively recovered, the product, although consisting mainly of *l*-mandelic acid, also contains some of the racemic acid, so that it would appear probable that the hydrochloric acid has the effect of racemising as well as of hydrolysing amygdalin. Moreover, it was found that on fractionally hydrolysing *iso*amygdalin the first fraction of mandelic acid was dextrorotatory, agreeing with the view that the dextro-glucoside is hydrolysed more readily than the lævo-isomeride, but on completing the hydrolysis the last fraction of mandelic acid was still dextrorotatory, although to a less extent than in the first portion. If racemisation did not occur, one would naturally have expected the last fraction to be lævorotatory, as was the case in the fractional hydrolysis of amygdalinic acid.

There is a great difference between the melting points of amygdalin and *iso*amygdalin—the former substance melting at 216°, the latter indefinitely between 125° and 140°. Wöhler (*Annalen*, 1842, **41**, 155) showed that ordinary amygdalin, solidified after melting, melts again between 125° and 130°, that is, at about the same temperature as *iso*-amygdalin. It therefore seemed probable that the transformation of amygdalin into the *iso*-compound could be effected by heat as well as by the action of alkalis. This view was confirmed by showing that, on hydrolysis, Wöhler's "amorphous amygdalin" gave mainly inactive mandelic acid. The compound was prepared by heating amygdalin at 230° in a narrow test-tube contained in an oil-bath. The substance melts to a viscid, brown liquid, which sets on cooling to a hard, resinous mass. It was found, on examining the mandelic acid obtained from the hydrolysis of different preparations, that if the amygdalin were only just fused and then immediately cooled, the mandelic acid was still lævorotatory to some extent, but, if on the other hand, the amygdalin were kept fused for several minutes, racemisation of the mandelic acid portion of the molecule was complete, and, as in the case of *iso*amygdalin, the mandelic acid resulting from hydrolysis was slightly dextrorotatory.

Some of the foregoing transformations of amygdalin are shown in the following diagram:



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THE FORMATION OF PRUSSIC ACID BY THE OXIDATION OF ALBUMINS.

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Introduction. Wishing to estimate the amount of hydrochloric acid contained in certain tumours, I employed the method recently published by A. Neumann[1] for converting albumins into ash. This method consists in heating the albuminous matter with a mixture of equal volumes of concentrated nitric and sulphuric acids and collecting the hydrochloric acid evolved in excess of a standard solution of silver nitrate which is afterwards titrated with sulphocyanate solution in the usual way.

On carrying out this process a difficulty was experienced in the titration as the solution obtained was always more or less yellow in colour and concordant results could not be obtained. The precipitate was therefore weighed, but higher results were now obtained when the solution was filtered directly without boiling to remove nitrous acid than when the solution was boiled. This difference in results was found to be due to the presence of silver cyanide in the precipitate of silver chloride; the silver cyanide was decomposed by the boiling acid solution with the evolution of prussic acid, and when this was again collected in silver nitrate solution it was found that a constant amount was formed.

These observations then led me to an investigation upon the action of boiling dilute nitric acid upon silver cyanide; freshly precipitated silver cyanide was found to be quantitatively converted into prussic acid. A simple method of separating and estimating silver cyanide and silver chloride was thus obtained[2].

Various albumins were now oxidised with this acid mixture and the amount of prussic acid evolved was estimated; each albumin gave a fairly constant amount which was different in the several cases.

Investigations were then made upon the origin of the prussic acid.

The albumin was hydrolysed with dilute sulphuric acid and the products so obtained were oxidised in the same way; the same amount of prussic acid was again formed.

Since the products of hydrolysis of albumin consist mainly of monoamino- and diamino-acids, certain of these were oxidised in order to determine, if possible, from which of them the prussic acid was formed. Only a small quantity of prussic acid was obtained which was by no means sufficient to account for the amount obtained from the albumin itself or its products of hydrolysis.

From these results it seemed as if the amino-groups played only a small part in the formation of prussic acid. In order to prove this supposition the products of hydrolysis were first treated with nitrous acid so as to destroy the amino-groups and then oxidised. The same amount of prussic acid was again obtained. It may therefore be assumed that the prussic acid originates from a decomposition product which does not contain nitrogen in the form of amino-groups.

The formation of prussic acid by the oxidation of albumins has been previously observed. In 1846 Schlieper[3] oxidised gelatine with chromic acid in order to confirm the statement made by Persoz and Marchand that prussic acid was formed in this way; two years later Guckelberger[4] showed that other albumins when oxidised with chromic acid also gave prussic acid. No estimation, however, of the amount of prussic acid evolved was made by these observers, nor has the formation of prussic acid been subsequently investigated. The amount of prussic acid formed by the oxidation of albumin with chromic acid was therefore estimated and it was found that it was the same as when the oxidation was performed with the nitric acid mixture.

The formation of prussic acid by the action of other oxidising agents has also been investigated. Manganese dioxide and dilute sulphuric acid was employed by Guckelberger with a negative result which I can entirely confirm.

Though albumins have often been oxidised with potassium permanganate the formation of prussic acid does not seem to have been observed. By the action of this oxidising agent in acid solution I obtained only a very small amount of prussic acid.

It was suggested by Kutscher and Steudel[5] that prussic acid might be formed by the oxidation with concentrated sulphuric acid in order to account for the low results which they obtained by Kjeldahl's method; I therefore made experiments with concentrated sulphuric acid but the presence of prussic acid could not be detected.

As described by Neumann the apparatus, in which the oxidation is carried out when the estimation of hydrochloric acid is required, consists of a $\frac{1}{2}$ litre retort into the tubulus of which a dropping funnel is ground. The neck of the retort is narrowed so that it passes into a flask containing excess of a standard solution of silver nitrate which is kept cold by immersion in a basin of cold water; the end of the retort is placed about 1 cm. above the level of the liquid in the flask which is filled to about a quarter of its volume. The albumin is then oxidised by heating it with a mixture of equal volumes of water, concentrated nitric acid and concentrated sulphuric acid, which is allowed to run in slowly through the tap-funnel until a white precipitate is no longer formed in the silver nitrate solution.

This apparatus was in the first place slightly modified by the employment of a distilling flask of the same capacity as the retort into the neck of which the tap-funnel was securely fastened by means of a cork. Experiments were then made to determine the accuracy of the results by this procedure with a known quantity of a standard solution of sodium chloride. The results were accurate. The same process was repeated with the addition of one gram of starch in order that organic matter might also be present; the same results were obtained. Although the cork is attacked by the acid fumes evolved during the process, this does not interfere with the results of the determination. This apparatus was consequently employed and the cork renewed for each experiment.

When the albuminous matter under investigation was treated in exactly the same way the reaction was at first very violent and large quantities of oxides of nitrogen were evolved; these gases on coming into contact with the silver nitrate solution produced a white precipitate; an early formation of silver chloride was also observed by Neumann.

Since there might be a small loss of hydrochloric acid during this violent reaction the process of carrying out the oxidation was further modified by passing the evolved gases through a condenser, and then through the solution of silver nitrate contained in a Volhard receiver attached to the end of the condenser. The solution, when treated in the manner described by Neumann, was then found to be more or less yellow in colour; this rendered the subsequent titration with sulphocyanate solution exceedingly difficult, and concordant results could not be obtained, so that the precipitate of silver chloride was weighed.

Experiments were first performed to determine whether this yellow substance was formed by the action of the acid mixture upon gelatine

and albumin; Von Fürth[6] in his publication upon the action of nitric acid on albumins makes no mention of the formation of a volatile nitro-derivative; his investigations were made chiefly upon xanthoprotein and its decomposition products. Both from gelatine and egg albumin the distillate was yellow, especially in the case of egg albumin; this colour became more intense on neutralising the acid solution with soda. The distillate also possessed the peculiar smell of aldehydes; their presence was verified by testing with ammoniacal silver nitrate solution which was strongly reduced. The aldehydes were then separated by the distillation of the neutral solution; the new distillate possessed the same smell but in addition that of prussic acid was noticeable; its presence was verified by the formation of a precipitate of Prussian blue when the solution was boiled with ferrous salt and alkali in the usual way and then acidified; on adding silver nitrate solution and acidifying silver cyanide was precipitated which gave the following analysis:

0·2328 grm. AgCN gave 0·1870 grm. Ag = 80·33 per cent. Ag.
AgCN requires 80·56 per cent. Ag.

The formation of prussic acid by the oxidation of albumins with the acid mixture was then investigated since its presence would cause a serious error in the estimation of chlorides by this method. As material gelatine was first employed, the process being carried out as described before, the silver precipitate was filtered off on to a tared filter without boiling the solution, washed with cold water, dried at 100° C., and weighed. The following three results were obtained:

Gelatine taken	Ag salt	p.c. Ag salt
5·2536 grms.	0·0536 grm.	1·02
5·3106	0·0546	1·03
4·5500	0·0532	0·96

Experiments were now made in which the solution was boiled before the silver salt was filtered off; three experiments were again performed:

Gelatine taken	Ag salt	p.c. Ag salt
7·0036 grms.	0·0296 grm.	0·42
5·7824	0·0186	0·32
7·5924	0·0214	0·28

From these results it will be seen that a loss had occurred. In order to confirm them a series of six experiments was carried out with dried egg albumin in exactly the same way; in the first three experiments the silver precipitate was weighed without boiling the solution, in the

remaining three experiments the solution was boiled before filtering off the precipitate.

The results were as follows:

	Albumin taken	Ag salt	p.c. Ag salt		Albumin taken	Ag salt	p.c. Ag salt
(1)	5·4090 grms.	0·2984 grm.	5·52	(4)	18·6982 grms.	0·6058 grm.	3·24
(2)	7·9336	0·4302	5·42	(5)	17·1636	0·5566	3·24
(3)	11·0812	0·6136	5·54	(6)	18·9408	0·6256	3·30

Just as in the case of gelatine a loss of silver salt had occurred. The solution was therefore distilled and the distillate after passing through a condenser was collected in a Volhard receiver containing silver nitrate solution. A white flocculent precipitate was formed which was filtered off, washed with cold water, dried at 100° C., and weighed; on analysis this substance was found to be silver cyanide. The silver chloride remaining in the solution was also estimated; the following results were obtained:

Albumin taken	AgCl	AgCN	p.c. AgCl	p.c. AgCN	Total p.c. Ag salts
13·6112 grms.	0·4538 grm.	0·2830 grm.	3·33	2·08	5·41
18·7718	0·6078	0·3896	3·24	2·07	5·31
17·2912	0·5536	0·3812	3·20	2·21	5·41

0·2778 grm. AgCN gave 0·2224 grm. Ag = 80·06 per cent. Ag.
0·3274 ,, 0·2622 ,, = 80·09 ,,

This behaviour of silver cyanide with boiling dilute nitric acid was then investigated: freshly precipitated silver cyanide was found to be converted into the theoretical amount of prussic acid by boiling dilute nitric acid; it can thus be separated from silver chloride and directly estimated [2].

As silver cyanide can thus be so easily separated from silver chloride the formation of prussic acid from other albumins and again from egg albumin and gelatine was investigated and its amount estimated. The amount of hydrochloric acid contained in them was also determined. In all cases the process was carried out in the following way:

The albumin was weighed out from a bottle into the distilling flask into which the dropping funnel was securely fastened; the flask was then connected to the condenser and to the end of the condenser a Volhard receiver containing excess of silver nitrate solution was attached. About 20 c.c. of the acid mixture were run in through the funnel and the liquid gently heated; after a short time a very violent reaction commenced with the evolution of large quantities of nitrous fumes which on coming into contact with the silver nitrate solution produced

a white precipitate. As soon as this violent reaction had subsided more acid mixture was slowly dropped in and the solution again heated; this was continued until evolution of gas no longer occurred and the solution in the flask became almost colourless when the oxidation was complete. The distillate was at first colourless but it contained a few oily drops; towards the end it became yellow in colour when a small quantity of a white solid also passed over. In order to separate the silver cyanide and silver chloride the solution and precipitate were carefully transferred into another flask and again distilled; the distillate was again collected in a solution of silver nitrate contained in a Volhard receiver. The silver cyanide so obtained was filtered off on to a tared filter, washed with cold water, then with hot water and hot alcohol so as to remove the white solid which also passed over, dried at 100° C. and weighed. The silver chloride which remained undecomposed was also filtered off, dried at 100° C. and weighed. The solution in which it was contained remained yellow in colour as the yellow substance did not again distil over except to a very slight extent. Its isolation and characterisation are still under investigation.

In the case of gelatine the reaction was at first by no means so violent as with the other albumins but a large amount of gas was also evolved; the distillate was also less deep yellow in colour.

The following tables show the results which were obtained; the silver cyanide was also analysed and the results of these determinations are appended:

Casein (Merck).

Amount taken	AgCl	AgCN	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
grms.	grm.	grm.						
5·1904	0·0166	0·1742	0·32			3·36		
4·1184	0·0102	0·1380	0·25			3·35		
6·3010	0·0118	0·2348	0·19	mean		3·73	mean	
6·1746	0·0186	0·2538	0·30	=0·27	0·07	4·11	=3·70	0·74
6·0514	0·0176	0·2234	0·29			3·69		
3·7936	0·0108	0·1506	0·28			3·97		

0·2842 grm. AgCN gave 0·2280 grm. Ag=80·22 per cent. Ag.
0·2706 ,, 0·2164 ,, =79·97 ,,

Hæmoglobin (Merck).

Amount taken grms.	AgCl grm.	AgCN grm.	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
6·5572	0·1160	0·2144	1·77			3·27		
3·3132	0·0564	0·0836	1·70			2·52		
4·5020	0·0790	0·1296	1·75	mean		2·88	mean	
5·3214	0·0930	0·1420	1·75	=1·74	0·44	2·67	=2·79	0·56
6·1670	0·1022	0·1590	1·66			2·58		
6·4946	0·1162	0·1816	1·79			2·80		

0·2044 grm. AgCN gave 0·1630 grm. Ag=79·75 per cent. Ag.
0·2076 ,, 0·1664 ,, =80·15 ,,

Fibrin (Merck).

Amount taken grms.	AgCl grm.	AgCN grm.	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
3·5612	0·0116	0·1128	0·33			3·17		
4·3014	0·0172	0·1466	0·40			3·41		
5·7578	0·0212	0·1918	0·37	mean		3·33	mean	
6·4052	0·0244	0·1934	0·38	=0·37	0·09	3·02	=3·26	0·66
5·1420	0·0200	0·1762	0·39			3·43		
4·6266	0·0170	0·1470	0·37			3·18		

0·3312 grm. AgCN gave 0·2644 grm. Ag=79·83 per cent. Ag.
0·2920 ,, 0·2336 ,, =80·00 ,,

Witte's Peptone.

Amount taken grms.	AgCl grm.	AgCN grm.	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
3·5364	0·0230	0·0860	0·65			2·43		
5·2290	0·0344	0·1474	0·66			2·82		
6·0860	0·0404	0·1692	0·66	mean		2·78	mean	
6·6184	0·0448	0·2068	0·68	=0·65	0·16	3·12	=2·61	0·53
6·0264	0·0374	0·1366	0·62			2·27		
4·2068	0·0256	0·0950	0·61			2·26		

0·2248 grm. AgCN gave 0·1798 grm. Ag=79·98 per cent. Ag.
0·3696 ,, 0·2970 ,, =80·36 ,,

Egg albumin.

Amount taken grms.	AgCl grm.	AgCN grm.	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
5·2420	0·1540	0·1454	2·94			2·77		
6·5108	0·1958	0·2366	3·01			3·63		
3·7760	0·1062	0·1218	2·81	mean		3·23	mean	
4·5496	0·1380	0·1108	3·03	=2·94	0·74	2·44	=2·98	0·60
5·2030	0·1498	0·1508	2·88			2·90		
6·5164	0·1936	0·1910	2·97			2·93		

0·3366 grm. AgCN gave 0·2702 grm. Ag=80·27 per cent. Ag.
0·1358 ,, 0·1086 ,, =79·97 ,,

Gelatine.

Amount taken	AgCl	AgCN	p.c. AgCl		Mean p.c. HCl	p.c. AgCN		Mean p.c. HCN
grms.	grm.	grm.						
6·6316	0·0054	0·0616	0·08			0·93		
10·7544	0·0122	0·1082	0·11			1·01		
13·5518	0·0146	0·1210	0·11	mean		0·89	mean	
20·1504	0·0254	0·1946	0·13	=0·12	0·03	0·97	=0·98	0·20
21·5628	0·0292	0·2118	0·13			0·98		
21·3944	0·0334	0·2386	0·16			1·11		

0·3076 grm. AgCN gave 0·2462 grm. Ag=80·04 per cent. Ag.
0·2148 ,, 0·1716 ,, =79·89 ,,
AgCN requires 80·56 per cent. Ag.

From these results it will be seen that the amount of silver chloride obtained is constant, and that Neumann's method gives accurate values when performed in this manner. For the simple estimation of chlorides it is only necessary to boil the solution containing the silver salts for about half-an-hour when all the silver cyanide will be decomposed. Neumann only heated the solution for 5—10 minutes to remove the nitrous acid, a time which is not sufficient to destroy all the silver cyanide.

The amount of silver cyanide, however, varied slightly; this can only be accounted for by the violence of the reaction which occurred at the commencement; the higher values were obtained when the acid mixture was heated very gently so that the reaction commenced as slowly as possible. The analyses of the silver cyanide were also slightly low; this may be due to the presence of a small quantity of the white solid which also distilled over, and which was not completely removed by washing with boiling alcohol, but it is more probable that it was due to a small quantity of another silver salt which adhered to the filter paper and turned black when exposed to the light. Volatile fatty acids and aldehydes were also contained in the distillate.

Oxidation with chromic acid. The volatile products formed by the oxidation of casein and other albumins with chromic acid and manganese dioxide were investigated by Guckelberger[4] in 1848 and were found to consist of prussic acid, aldehydes, fatty acids and nitriles. No attempt however was made by this observer to estimate the prussic acid; experiments were therefore undertaken to estimate its amount and compare it with the amount obtained with the acid mixture.

A known quantity of casein was oxidised with twice its weight of potassium dichromate, three times its weight of sulphuric acid and

thirty times its weight of water in the same apparatus as previously described, the sulphuric acid diluted with the water being slowly added to the finely powdered potassium dichromate mixed with the casein. The solution was gently heated at first as frothing occurred, but this soon ceased and the distillation could be carried on. Only a very small amount of precipitate was found in the receiver containing silver nitrate solution acidified with nitric acid; when redistilled this again passed over but its amount was too small for further investigation.

Guckelberger stated that these proportions of albumin, potassium dichromate, sulphuric acid and water were the best to obtain a good yield of volatile products; a larger amount of prussic acid was therefore expected.

Two years previously Schlieper[3] had oxidised gelatine with chromic acid in the proportions of 1 part of gelatine, 4 parts of potassium dichromate, $7\frac{1}{2}$ parts of sulphuric acid and 25 parts of water and had obtained prussic acid. Experiments were therefore made to determine whether a larger amount of prussic acid was obtained when these proportions were used; the same procedure as before was adopted and the distillate again distilled to separate the silver cyanide; the results were:

Casein taken	AgCN	p.c. AgCN	p.c. HCN	
4·3148 grms.	0·1838 grm.	4·26	0·85	mean =0·74
5·2886	0·1840	3·48	0·70	
4·1608	0·1558	3·75	0·75	
4·4182	0·1838	3·39	0·68	

0·1708 grm. AgCN gave 0·1368 grm. Ag = 80·09 per cent. Ag.
0·3336 ,, 0·2676 ,, = 80·22 ,,

The same amount of prussic acid is thus formed by the oxidation of casein with suitable proportions of chromic acid as with the nitric acid mixture.

Oxidation with manganese dioxide. Guckelberger[4] also oxidised albumins with manganese dioxide and sulphuric acid; with this oxidising agent he found that prussic acid was not contained amongst the volatile products.

A known quantity of casein was oxidised with the same proportions of manganese dioxide, sulphuric acid and water as employed by Guckelberger, and the distillate was collected in silver nitrate solution as in all the previous experiments. The distillation had to be carried out at first very carefully as there was a great deal of frothing at the com-

mencement, but this ceased after a short time and the distillation could be proceeded with. After 3—4 hours' distillation no precipitate of silver cyanide was observed and Guckelberger's result was confirmed.

Oxidation with potassium permanganate. Though many experiments have been performed on the oxidation of albumins with potassium permanganate, no mention is made whether prussic acid is formed; investigations were therefore carried out for this purpose with acid permanganate, which was employed in such a quantity as corresponded in oxidising power to that of the chromic acid previously used.

A known weight of casein was mixed with $2\frac{1}{2}$ times its weight of finely powdered potassium permanganate, and to this mixture, contained in the same apparatus as in the previous experiments, $7\frac{1}{2}$ times its weight of sulphuric acid mixed with 25 times its weight of water was slowly added. A very violent reaction commenced, with a great deal of frothing and spurting, so that a larger flask was employed; then also it was very difficult to prevent a little of the solution being splashed into the condenser and carried over into the silver nitrate solution. When this reaction ceased the rest of the sulphuric acid was slowly added and distillation commenced; it was continued for 2—3 hours. A small quantity of precipitate was formed, and on redistillation in the usual way a small precipitate of silver cyanide was obtained:

Casein taken	AgCN	p.c. AgCN
4·4332 grms.	0·0044 grm.	0·10
5·1860	0·0086	0·16
6·0986	0·0098	0·16

0·0194 grm. AgCN gave 0·0152 grm. Ag. = 78·35 per cent. Ag.

The small quantity of silver cyanide obtained by oxidation with permanganate is not pure as seen from its analysis.

Oxidation with concentrated sulphuric acid. Kutscher and Steudel[5] suggested that prussic acid might be formed by Kjeldahl's method for estimating nitrogen so as to explain the low results which they obtained; they made a few experiments but could not detect the presence of prussic acid.

A known quantity of casein was oxidised by heating it with concentrated sulphuric acid in the usual way; the gases evolved, when passed into silver nitrate solution, gave only a precipitate of silver sulphite. Two experiments were also made where the gases evolved were passed into excess of caustic soda solution, and the sodium sulphite so produced was then oxidised with potassium permanganate. The

solution was then acidified with nitric acid and distilled and the distillate collected in silver nitrate solution; in the first experiment only a slight turbidity was formed, and in the second experiment the silver nitrate solution remained clear. Thus, the oxidation with sulphuric acid caused no evolution of prussic acid.

Experiments were now made to determine the origin of the prussic acid; for this purpose it was firstly necessary to determine whether the hydrolytic decomposition products of albumin also gave rise to it and in what amount.

A known quantity of albumin was hydrolysed by boiling with three times its weight of concentrated sulphuric acid and six times its weight of water under a reflux condenser for 14 hours [7]; in the first experiment the end of the condenser was connected by a tube to a Volhard receiver containing silver nitrate solution so as to determine whether prussic acid was formed during hydrolysis; only a very small quantity of a dark silver salt, in all probability silver chloride, was present in the solution at the end of the hydrolysis so that prussic acid is not formed during hydrolysis. The solution containing the products of hydrolysis was then oxidised with the acid mixture in the same way as described above. Here, however, the acid mixture at the commencement had to be added very slowly as frothing occurred; this ceased after a short time and the solution gradually became concentrated by distillation. When the concentration had become sufficient, oxidation suddenly commenced with the evolution of nitrous fumes and at the same time a white precipitate was formed in the receiver; only a small quantity of precipitate had been formed previous to the commencement of the oxidation. Just as with the albumins themselves, the oxidation was then completed and the distillate, which was again yellow, redistilled to separate the silver salts, both of which were estimated as before:

Casein.

Amount taken	AgCl	AgCN	p.c. AgCl	p.c. AgCN	p.c. HCN
4·3078 grms.	0·0096 grm.	0·1222 grm.	0·22 } mean	2·84 } mean	
5·4528	0·0160	0·1468	0·29 } =0·26	2·69 } =2·77	0·56

0·2596 grm. AgCN gave 0·2074 grm. Ag=79·89 per cent. Ag.

Hæmoglobin.

4·1834	0·0712	0·0940	1·70 } mean	2·25 } mean	
5·4010	0·0980	0·1170	1·81 } =1·76	2·17 } =2·21	0·45

0·1984 grm. AgCN gave 0·1586 grm. Ag=79·94 per cent. Ag.

Fibrin.

Amount taken	AgCl	AgCN	p.c. AgCl	p.c. AgCN	p.c. HCN
4·3602 grms.	0·0186 grm.	0·1112 grm.	0·43 } mean	2·55 } mean	
5·3106	0·0176	0·1336	0·33 } =0·38	2·52 } =2·54	0·51

0·2346 grm. AgCN gave 0·1874 grm. Ag = 79·88 per cent. Ag.

Witte's Peptone.

4·6244	0·0308	0·1084	0·67 } mean	2·34 } mean	
5·4470	0·0388	0·1234	0·71 } =0·69	2·26 } =2·30	0·46

0·2248 grm. AgCN gave 0·1800 grm. Ag = 80·07 per cent. Ag.

Egg albumin.

4·5812	0·1022	0·1464	2·23 } mean	3·20 } mean	
5·8514	0·1564	0·2210	2·67 } =2·45	3·78 } =3·49	0·70

0·2172 grm. AgCN gave 0·1742 grm. Ag = 80·20 per cent. Ag.

Gelatine.

4·6360	0·0086	0·0400	0·19 } mean	0·86 } mean	
7·0608	0·0108	0·0394	0·15 } =0·17	0·56 } =0·69	0·14
8·1854	0·0132	0·0532	0·16 }	0·65 }	

0·1184 grm. AgCN gave 0·0942 grm. Ag = 79·56 per cent. Ag.

Thus, prussic acid is formed in nearly the same amount from the products of hydrolysis as from the albumin itself.

Certain monoamino-acids and other nitrogen-containing compounds were then oxidised to determine whether the prussic acid originated from them. The experiments were carried out in the same way as described for the albumins; but the oxidation, except in the case of tyrosine, was by no means so violent at the commencement, the reaction only occurring when the acid mixture had become concentrated by distillation. With tyrosine, however, a very violent reaction occurred when the acid mixture was added to it, no heating being necessary; the reaction ceased after a short time and the oxidation was completed in the usual way. The distillate from tyrosine was yellow like that from the albumins; the volatile nitro-derivative thus appears to be derived from tyrosine, but from the fact that it is also obtained from gelatine which contains no tyrosine, phenylalanine[8], the only aromatic derivative as yet isolated from it, must also be concerned in its formation. The following compounds were investigated with the results given below:

Glycocoll.

Amount taken	AgCN	p.c. AgCN
6·3140 grms.	0·0096 grms.	0·15
5·2104	0·0066	0·13
6·2168	0·0058	0·09

The quantity obtained was unfortunately too small for analysis; in appearance it was not like silver cyanide and it turned black in the light.

Alanine.

Amount taken	AgCN	p.c. AgCN	p.c. HCN
4·0364 grms.	0·0290 grm.	0·72	0·15 } mean
5·8762	0·0384	0·65	0·13 } =0·15
6·6556	0·0546	0·82	0·17 }

0·1484 grm. AgCN gave 0·1178 grm. Ag=79·38 per cent. Ag.

α-Pyrrolidinecarboxylic acid (obtained from gelatine[8]).

1·2908	0·0144	1·11	0·23 } mean
1·2760	0·0138	1·08	0·22 } =0·22

0·0158 grm. AgCN gave 0·0122 grm. Ag=77·21 per cent. Ag.

Aspartic acid.

4·3058	0·0158	0·37	0·08 } mean
5·2392	0·0164	0·31	0·06 } =0·07

0·0262 grm. AgCN gave 0·0206 grm. Ag=78·63 per cent. Ag.

Tyrosine.

1·1406	0·0462	4·05	0·81 } mean
0·5850	0·0198	3·38	0·68 } =0·79
1·2380	0·0546	4·41	0·88 }

0·0844 grm. AgCN gave 0·0670 grm. Ag=79·38 per cent. Ag.

Asparagine.

4·8390	0·0258	0·53	0·11 } mean
5·4156	0·0132	0·24	0·05 } =0·08

0·0304 grm. AgCN gave 0·0242 grm. Ag=79·60 per cent. Ag.

Urea.

5·6122	0·0006	0·01
7·1160	0·0006	0·01

The quantity of silver salt was too small for analysis; it became black on exposure to the light.

Guanidine nitrate.

Amount taken	AgCN	p.c. AgCN	p.c. HCN
5·0334 grms.	0·0012 grm.	0·02	0·01 } mean
7·8024	0·0052	0·07	0·01 } = 0·01

The quantity of silver salt was again too small for analysis; it behaved like that from urea.

Succinimide.

5·2388	0·0310	0·59	0·12 } mean
5·8432	0·0332	0·57	0·11 } = 0·12

0·0580 grm. AgCN gave 0·0462 grm. Ag = 79·65 per cent. Ag.

Thus only in the case of tyrosine an amount of prussic acid is obtained which corresponds to that given by the albumins; this amount, however, is not sufficient to account for the total amount given by the albumin, as only about 5 per cent. of tyrosine has been obtained as the maximum yield on hydrolysis. These values have been collected together by Reach[9].

Since the greatest amount of arginine is contained in gelatine, of histidine in hæmoglobin, and of lysine in casein and gelatine, it may be assumed that these hexone bases are not the origin of the prussic acid, for these albumins should then give the greatest quantity of prussic acid, which is not the case. Prussic acid has been observed by Zickgraf[10] among the products of the oxidation of lysine with permanganate, and Herzog[11] has observed its formation from histidine by oxidation. Froehde[12] obtained prussic acid by the oxidation of tyrosine with chromic acid but made no estimation of its amount. Unfortunately the amount of tyrosine available was insufficient for a repetition of Froehde's experiments in order to estimate the amount of prussic acid evolved by this method of oxidation. Experiments are in progress on this point.

Many complex organic compounds when oxidised with nitric acid also give rise to prussic acid; Hantzsch[13] has explained this reaction by assuming that the nitrous acid, which is first formed, acts on the first oxidation product forming an oximido-derivative; these compounds (diisonitrosoacetone, oximidoacetic acid, isonitrosomalonic acid) which contain the $= N - OH$ group are decomposed by boiling with water with the evolution of prussic acid. This explanation cannot be applied to the albumins since, as has been shown, the same amount of prussic acid is formed by the oxidation with chromic acid.

In order to show that the products of hydrolysis of albumin which contain amino-groups were not the mother substances of the prussic acid although they take a share in its formation, a known quantity of egg albumin was hydrolysed as before and to the cold solution a solution of sodium nitrite was slowly added so as to destroy the amino-groups: this solution contained an equivalent amount of sodium nitrite to that employed by Jochem[14] in his investigations. The solution so obtained was then gently heated to remove the excess of nitrous acid and slightly concentrated by distillation, the distillate being collected in silver nitrate solution. It was then oxidised with the acid mixture and the same procedure carried out as in the previous experiments; the same quantity of prussic acid was again obtained:

4·5456 grms. egg albumin gave 0·1322 grm. AgCN = 2·91 per cent. AgCN
= 0·59 per cent. HCN.
0·1264 grm. AgCN gave 0·1014 grm. Ag = 80·22 per cent. Ag.

During the heating to remove the excess of nitrous acid it was noticed that a white precipitate was formed in the silver nitrate solution; two experiments were made where this precipitate was collected separately before the solution was oxidised with the acid mixture; the receiver was changed when the oxidation was commenced and a further quantity was then obtained. In both cases the separation of silver cyanide from silver chloride was then carried out with the following results:

Egg albumin taken	AgCN I	p.c. AgCN I	AgCN II	p.c. AgCN II	Total p.c. AgCN	p.c. HCN
6·8194 grms.	0·1376 grm.	2·02	0·1190 grm.	1·74	3·76	0·75 } mean
5·0682	0·0728	1·44	0·0912	1·80	3·24	0·65 } = 0·70

0·2028 grm. AgCN I gave 0·1624 grm. Ag = 80·08 per cent. Ag.
0·2020 ,, II ,, 0·1620 ,, = 80·20 ,,

Prussic acid is thus formed from the products of hydrolysis of albumin after treatment with nitrous acid in the same amount as when they are not so treated; a portion is evolved when the solution containing nitrous acid is heated.

Summary.

By the oxidation of albumins with Neumann's acid mixture prussic acid is evolved; its amount is constant and varies for each albumin.

The same quantity of prussic acid is formed from the products of hydrolysis of albumin when they are oxidised.

Only a small quantity of prussic acid is formed from the amino-acids by oxidation.

The same amount of prussic acid is evolved after the products of hydrolysis have been treated with nitrous acid so that the amino-groups play only a small part in its formation.

Oxidation with potassium dichromate and sulphuric acid in suitable proportions gives rise to the same amount of prussic acid.

Oxidation with manganese dioxide and sulphuric acid, potassium permanganate and sulphuric acid, and concentrated sulphuric acid does not give rise to prussic acid or in very small amount only.

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THE FORMATION OF PRUSSIC ACID BY THE OXIDATION OF ALBUMINS. II.

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In a previous communication [1] it was shown that, when albumins were oxidised with Neumann's nitric acid mixture, a constant amount of prussic acid was formed. Oxidation was also carried out with other oxidising agents; of these, only chromic acid gave rise to the formation of prussic acid which in the case of casein was the same in amount as when oxidation was performed with the nitric acid mixture.

Investigations which were then made to determine the origin of the prussic acid showed that of the hydrolytic decomposition products of albumins only tyrosine gave an amount corresponding to that given by the albumins themselves when it was oxidised with the nitric acid mixture. Since, both with the nitric acid mixture and with chromic acid, the same amount of prussic acid was formed when casein was oxidised, it was expected that its origin would be the same in the two cases especially as Froehde [2] in 1860 had obtained prussic acid by the oxidation of tyrosine with chromic acid. Experiments however did not justify this assumption, since tyrosine when oxidised with chromic acid did not yield prussic acid, a result which confirms that of Thudichum and Wanklyn [3] in 1869.

Inquiry as to the origin of the prussic acid when chromic acid was employed as oxidising agent was thus necessitated, and it was found that glycocoll and aspartic acid were the substances from which it originated, although a very small quantity was obtained from other albumins.

These results led to a determination of the amount of prussic acid evolved from the other albumins; as expected it was quite different from that obtained when the nitric acid mixture was employed.

Although the first experiments were carried out with tyrosine it is more convenient to describe and give the results in the same order as when the oxidation was carried out with the nitric acid mixture.

The same apparatus and the same procedure as in the previous experiments were employed for the determination of the prussic acid; instead, however, of mixing the substance under examination with the finely powdered potassium dichromate and then adding the dilute sulphuric acid, the potassium dichromate was dissolved in the sulphuric acid and water and this solution was added to the substance in a distilling flask. The proportions employed were those of Schlieper, namely, 1 part of albumin, 4 parts of potassium dichromate, 25 parts of water and 7·5 parts of sulphuric acid. At the commencement of the distillation no frothing occurred but towards the end the mixture bumped rather violently owing to the separation of salts; as a white precipitate was still being formed in the receiver more water was added in several small portions through the tap funnel; this put an end to the bumping and the distillation could be carried on until a precipitate was no longer formed in the silver nitrate solution. Before redistilling the contents of the receiver in order to separate the silver cyanide from any silver chloride which might also be present, nitric acid was added so as to raise the acidity of the solution to about four times normal. As before the silver cyanide obtained was collected on a tared filter-paper, dried at 100° C. and weighed.

The following tables show the results which were obtained; for comparison the mean results obtained when oxidation was carried out with the nitric acid mixture are also given. The silver cyanide was also analysed and the results of these determinations are appended.

Casein (Merck).

Amount taken grms.	AgCN grm.	% AgCN		Mean % HCN	HNO_3 mixture mean % HCN
4·1172	0·1702	4·13	mean		
5·0794	0·2028	3·99	=4·05	0·82	0·74
4·7646	0·1926	4·04			

0·1558 grm. AgCN gave 0·1254 grm. Ag=80·49 per cent. Ag
0·1940 ,, ,, ,, 0·1558 ,, =80·31 ,, ,,

Hæmoglobin (Merck).

Amount taken grms.	AgCN grm.	% AgCN		Mean % HCN	HNO_3 mixture mean % HCN
4·1298	0·2246	5·44	mean		
5·0048	0·2726	5·45	=5·60	1·13	0·56
4·7616	0·2818	5·90			

0·2214 grm. AgCN gave 0·1778 grm. Ag=80·31 per cent. Ag
0·2682 ,, ,, ,, 0·2160 ,, ,, =80·54 ,, ,,

Fibrin (Merck).

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN	HNO_3 mixture mean % HCN
5·2366	0·2848	5·44	mean	
5·0912	0·2770	5·44	=5·51	1·11	0·66
3·9678	0·2236	5·64		

0·1924 grm. AgCN gave 0·1546 grm. Ag=80·35 per cent. Ag
0·2816 ,, ,, ,, 0·2268 ,, ,, =80·54 ,, ,,

Witte's Peptone.

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN	HNO_3 mixture mean % HCN
5·1660	0·2294	4·44	mean	
4·2894	0·2026	4·72	=4·67	0·94	0·53
3·2036	0·1556	4·85		

0·2286 grm. AgCN gave 0·1836 grm. Ag=80·31 per cent. Ag
0·1972 ,, ,, ,, 0·1584 ,, ,, =80·32 ,, ,,

Egg albumin.

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN	HNO_3 mixture mean % HCN
4·2182	0·1816	4·30	mean	
3·1208	0·1360	4·36	=4·35	0·88	0·60
5·2332	0·2290	4·38		

0·1762 grm. AgCN gave 0·1418 grm. Ag=80·48 per cent. Ag
0·1576 grm. ,, ,, 0·1264 ,, ,, =80·20 ,, ,,

Gelatine.

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN	HNO_3 mixture mean % HCN
2·1046	0·3646	17·32	mean	
3·1112	0·4182	13·44	=13·62	2·75	0·20
2·4236	0·3138	12·95		
3·0412	0·3270	10·75		

0·2598 grm. AgCN gave 0·2088 grm. Ag=80·37 per cent. Ag
0·4094 grm. ,, ,, 0·3290 ,, ,, =80·36 ,, ,,

From these tables it will be seen that the amount of prussic acid obtained by oxidation with chromic acid is greater than by oxidation with the nitric acid mixture. It is especially noticeable in the case of gelatine where 10—15 times the quantity is evolved; in the case of the other albumins about double the quantity is formed except in that of casein where the amounts are practically the same. This result led previously to the supposition that the origin of the prussic acid would be the same substance—tyrosine—which on examination proved not to be the case.

As tyrosine was not the substance from which the prussic acid was formed experiments were made to determine whether the hydrolysis products of casein also gave rise to it. A known quantity of casein was therefore hydrolysed by boiling with three times its weight of concentrated sulphuric acid and six times its weight of water under a reflux condenser for 14 hours. The solution so obtained was oxidised by the same quantity of potassium dichromate dissolved in sulphuric acid and water in such amounts as to be the same as those used in the oxidation of the casein: this quantity was added to the solution and the distillation carried out as before, when the following results were obtained:

Amount taken grms.	AgCN grm.	% AgCN		Mean % HCN
5·3126	0·3358	6·32	mean = 6·21	1·25
5·2060	0·3072	5·90		
4·2136	0·2580	6·12		
6·0664	0·3944	6·50		

0·5168 grm. AgCN gave 0·4156 grm. Ag = 80·42 per cent. Ag
0·3880 grm. „ „ 0·3128 „ „ = 80·62 „ „

The amount of prussic acid thus obtained will be seen to be about half as great again as that obtained from the casein itself. This would appear to show that some of the groups which give rise to the prussic acid on oxidation are combined in the casein molecule but on hydrolysis are set free, thus giving rise to the larger amount of prussic acid.

Nearly the whole of the prussic acid is evolved from the mono-amino-acids, *i.e.* the products of hydrolysis not precipitated by phosphotungstic acid. Two experiments were made with 5 grms. of casein which were hydrolysed and then treated with phosphotungstic acid as long as a precipitate was formed. The filtrate and precipitate were then treated with baryta in the usual way to remove phosphotungstic acid and after acidifying with sulphuric acid and filtering from barium sulphate were oxidised by chromic acid. The products contained in the filtrate gave 0·8 and 1·0 per cent. of prussic acid, whereas the diamino-acids in the precipitate gave 0·1 and 0·05 per cent. of prussic acid.

The various mono-amino and diamino-acids were then oxidised in the same way as the albumins and with the same proportions of potassium dichromate, sulphuric acid and water. The following is a list of the compounds which were examined and the results which were obtained:

Glycocoll.

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN
0·5842	0·3372	57·72	
0·3548	0·1658	46·73	mean =55·05 11·10
0·2490	0·1352	54·30	
0·3684	0·2264	61·45	

0·4944 grm. AgCN gave 0·3976 grm. Ag=80·42 per cent. Ag
0·1328 ,, ,, ,, 0·1072 ,, ,, =80·72 ,, ,,

Alanine.

0·2722	—	—	—
0·6320	—	—	—

Leucine.

0·5172	0·0198	3·83	mean
1·0868	0·0366	3·37	=3·37 0·68
1·2014	0·0348	2·90	

0·0870 grm. AgCN gave 0·0694 grm. Ag=79·22 per cent. Ag

Tyrosine.

0·5050	—	—	—
0·7940	—	—	—

Tryptophane[1].

0·3466	—	—	—

Proline or α-Pyrrolidine Carboxylic Acid.

0·4202	0·0076	1·81	mean
0·5238	0·0098	1·87	=1·84 0·37

0·0154 grm. AgCN gave 0·0122 grm. Ag=79·22 per cent. Ag

Aspartic Acid.

0·5482	0·2034	37·10	mean
0·7624	0·2890	37·91	=38·15 7·70
0·5208	0·2054	39·44	

0·2002 grm. AgCN gave 0·1614 grm. Ag=80·60 per cent. Ag
0·2854 ,, ,, ,, 0·2296 ,, ,, =80·45 ,, ,,

Glutamic Acid.

0·4922	trace	—	—
0·4950	trace	—	—

[1] See note [1], p. 56.

Glucosamine Hydrochloride[1].

Amount taken grms.	AgCN grm.	% AgCN	Mean % HCN
0·8496	0·0032	0·38	0·08

Lysine Hydrochloride[1].

1·0588	0·0052	0·49	0·10

Arginine Carbonate[1].

0·6132	0·0036	0·59	0·12

[1] These compounds were kindly given to me by Messrs Hopkins, Cathcart, Dakin, and Hedin, to whom I express my best thanks.

From these results it is evident that glycocoll and aspartic acids are the mother substances of practically the whole of the prussic acid formed by the oxidation of albumins by chromic acid, the small amounts obtained from the other amino-acids being negligible in comparison.

The amounts of silver cyanide obtained by the oxidation of glycocoll are by no means constant; variable results were also obtained in the case of gelatine and they may be attributed to this constituent which is contained in gelatine to the extent of 16·5 per cent. They are probably due to the difficulty of distilling so small a quantity which it was necessary to employ in order to obtain a quantity of silver cyanide which could be easily manipulated, as there was always considerable bumping and fresh water had to be added many times before precipitation of silver cyanide in the receiver entirely ceased.

The formation of prussic acid from glycocoll was observed as early as 1849 by Liebig [4], who used manganese dioxide and sulphuric acid as the oxidising agent; its amount however was not estimated. I have not been able to find any other reference to the formation of prussic acid from glycocoll.

Aspartic acid, the other compound to which the origin of the prussic acid has been traced, gave much more concordant results; the highest figure was obtained when fresh water was added several times and the distillation prolonged over a much longer time. It is to this substance that the prussic acid owes its origin in the case of hæmoglobin which, according to the researches of E. Fischer and E. Abderhalden [5], does not contain glycocoll.

Prussic acid was not obtained from alanine or from tyrosine and in all probability phenyalanine will behave in a similar way. I was un-

fortunately unable to obtain a small quantity of this amino-acid in order to perform this experiment.

It was stated by Froehde[2] in 1860 that tyrosine on oxidation with chromic acid gave rise to prussic acid, a result which was not confirmed by Thudichum and Wanklyn[3] nine years later; these observers found that formic and carbonic acids were the only volatile oxidation products of tyrosine. Liebig[4] in his paper (*loc. cit.*) quotes experiments of Fr. Bopp who also found that tyrosine on oxidation gave no volatile products containing nitrogen. The prussic acid obtained by Froehde evidently arose from some leucine which he stated was contained in the tyrosine and to which he attributed the presence of valeric and acetic acids amongst the decomposition products.

Tryptophane also did not give rise to prussic acid and glutamic acid gave only a trace of a silver salt which behaved like silver cyanide on distillation with acid. The other amino-acids gave only very small amounts of prussic acid, which may here be regarded as a secondary product. According to Liebig and Guckelberger valeronitrile is the only volatile product containing nitrogen which is obtained from leucine, and is regarded as corresponding to the prussic acid or formonitrile obtained from glycocoll.

As regards the mode of formation of the prussic acid from glycocoll it may be supposed that the first product of the oxidation is nitroso-acetic acid, which according to Cramer[6] breaks down on heating with water at 120° C. into prussic acid, carbonic acid and water, thus:

$$CH_2.NH_2.COOH + 2O = CH = NOH.COOH + H_2O$$
$$CH = NOH - COOH = HCN + CO_2 + H_2O.$$

The formation of oxamic acid which has quite recently been observed by Kutscher and Schenk[7] to be an oxidation product of gelatine with calcium permanganate and has been attributed to the glycocoll which yields this compound on oxidation with permanganate can also be explained on the supposition that nitroso-acetic acid is the first oxidation product of glycocoll if, as v. Pechmann[8] suggested, this compound undergoes the Beckmann rearrangement into the isomeric acid amide, namely, oxamic acid:

$$CH = NOH.COOH \rightarrow CO.NH_2.COOH.$$

It is more difficult to explain the formation of prussic acid from aspartic acid; like the other amino-acids the NH_2 group in it is combined with a secondary carbon atom whereas in glycocoll it is attached to a primary carbon atom. Its homologue, glutamic acid,

only gave a trace of silver cyanide, and this renders it impossible to give a satisfactory explanation.

As regards the oxidation by nitric acid it was formerly stated that Hantzsch's explanation of the formation of prussic acid by the action of nitric acid on complex organic compounds could not be applied to the albumins; in the light, however, of the above experiments with chromic acid this explanation of Hantzsch's may be true for the formation of prussic acid from tyrosine by oxidation with nitric acid.

Summary.

By the oxidation of albumins with chromic acid a constant amount of prussic acid is evolved which varies for each albumin.

The amount of prussic acid obtained by oxidation with chromic acid is in general greater than by oxidation with Neumann's nitric acid mixture.

The products of hydrolysis of albumins give rise to a slightly greater amount of prussic acid than the albumins themselves.

The prussic acid arises from the glycocoll and aspartic acid but the other amino-acids also give rise to a small quantity which is negligible in comparison.

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Products of Urotryptic[1] digestion.

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The presence of various enzymes in the normal urine has been a recognised fact for a great number of years. The ferments generally acknowledged to be present are Diastase, Rennet, and more particularly Pepsin. This latter enzyme has had most conclusive proofs given as to its presence by Delezenne and Frouin (1) and more recently by Matthes (2), who showed that pepsin disappeared from the urine, although previously present, after complete removal of the Stomach. Corroborative evidence was also given by Leo and Senator (3) in the case of the professional fasting man Cetti, where these observers found that pepsin disappeared after the fast had continued for some time and reappeared when food was again taken.

The occurrence of the other great digestive ferment — Trypsin — in normal urine has however been much discussed, many being for and many against the idea that this ferment is excreted with the urine. Amongst those who maintain that trypsin, or at least a proteolytic enzyme acting in an alkaline medium, can be obtained from the urine are Sahli (4), Gehrig (5), Bendersky (6) and Tasully (7), whilst Hoffmann (8), Stadelmann (9), Leo (10) and Mees (11) deny its presence. Hopkins (12) found it in pathological but not in normal urine.

Owing to this diversity of opinion it was thought that an inquiry into the subject might be of interest. One was the more encouraged to undertake the work as owing to the use of a method employed by Hedin (13) in his work on the enzymes of the blood and spleen, the presence of an enzyme acting in many ways like Trypsin could be detected constantly in normal urine. It being now possible to obtain the enzyme without any great difficulty it was

1) N. B. All that is to be inferred from the use of the word Urotrypsin is that there is present in normal urine an enzyme or enzymes capable of acting in an alkaline medium i. e. have trypsin like properties.

decided to carry out a prolonged digestion on fibrin with the same in order to determine what the end products were.

That the resulting digestion was really due to an enzyme acting in alkaline medium, present in normal urine, is shewn by the fact that the Casein-enzyme precipitate (see later) left in sodium carbonate solution at 37° digests itself. The figures given below are the number of ccm of $\frac{N}{10}$ H_2SO_4 required to neutralise the NH_3, from the Kjeldahl nitrogen determination of 10 ccm of the filtrate obtained after precipitation of the digests with Tannic Acid.

	$\frac{N}{10}$ H_2SO_4
Before Digestion (Control.)	0,4 ccm
Digestion in 0.25 % Na_2CO_3 at 37°.	
3 days	2,1 „
6 „	4,5 „
10 „	5,1 „

Methods. The Urine, as fresh as possible, was first mixed with about an equal volume of distilled water (The dilution is necessary as the presence of salts etc. in too high concentration diminishs the amount of the enyzme taken down by the casein). To each litre of the mixture was then added 10 ccm of a 3½ % solution of Caseinogen in 0,25 % Sodium Carbonate. The Casein, after thorough mixing with the urine, was precipitated by 20 % Acetic acid (the quantity of acid necessary having been determined by previous experiment). After complete precipitation the clear supernatant fluid was siphoned off, and the precipitate was then transferred to a filter, where it was washed with distilled water until no acid reaction given. After complete washing the casein-enzyme combination was added to the fibrin in a large stoppered bottle and filled up with 0,25 % Sodium Carbonate solution.

The quantities used in this research were roughly 40 litres of urine, 2 Kilos. fresh moist fibrin and 7—8 litres Sodium Carbonate solution. The mixture was kept in the hot room at 37° for 7 months, that was until examination of small samples of the digest, taken at intervals of about 10 days, shewed no increase, determination by Kjeldahl's method, of the introgenous substances not precipitable by tannic acid. To prevent putrefaction both Toluol and chloroform were added to the digest.

As the resulting digest fluid contained very little insoluble matter in suspension, the whole was poured into a large basin, and after slightly acidifying with Acetic Acid heated for some time on the water-bath. The resultant precipitate was then filtered off. The filtrate, which gave a voluminous precipitate with Tannic Acid, was further treated for removal of all possible proteid with freshly precipitated lead hydroxide in excess. The lead left in solution was removed by means of Sulphuretted Hydrogen, the resulting filtrate from the lead sulphide being further concentrated. By these means one ob-

tained a fluid free from all substances precipitable by Tannic Acid. This filtrate in addition to giving a strong Biuret reaction, gave also that of Adamkiewicz, but none with Bromine water. Millon's reaction was likewise very well marked but no blackening of lead in alkaline solution was noted.

Separation of the Products of Digestion.

1. The solution was precipitated with a sufficient quantity of 10 % Mercuric sulphate solution in presence of Sulphuric Acid. Isolation of Tryptophane: Hopkins and Cole (14).

2. The excess of Mercury was removed from the filtrate of 1 by means of H_2S and to the filtrate from the Mercuric Sulphide was added a sufficient quantity of phosphotungstic. acid. Separation of Mono- and Diamino Acids.

I. Precipitate with Mercuric Sulphate.

This precipitate was thoroughly well washed after filtration with 5 % Sulphuric Acid, until the washings gave no red colouration with Millons reagent i. e. until all Tyrosine, which this agent can precipitate, had been washed out. The precipitate was then decomposed with H_2S in the presence of about 2 % H_2SO_4. This decomposition was repeated some 4—5 times at first in cold and afterwards in warm solution. The united filtrates from the above decompositions were heated on the water bath to remove the excess of H_2S, and then the whole once more precipitated with Mercuric Sulphate[1], and the subsequent decomposition with H_2S conducted as above described.

The resulting solution gave an exceptionally well marked Adamkiewicz reaction, and also the „reddish" colouration with Bromine water — the proteinchrome reaction — was now obtained; but no Millon reaction and no biuret. This solution from which the Sulphuric Acid had been exactly removed with Baryta, was concentrated on the water bath, in presence of 90 % Alcohol, to a very small volume. As it had become somewhat dark in colour is was treated with a little animal charcoal, and allowed to cool when is set to a thick mass of crystals. These were filtered off ac the pump, washed well with 90 % Alcohol, sucked dry, and after again treating with animal charcoal recrystallised several times from about 75 % Alcohol.

The crystals obtained were in the form of large glistening plates.

Analysis.

0,1064 grm Substance gave 0,2533 grm CO_2 and 0,0565 grm H_2O.
Found = 64,93 % C : 5.94 % H.

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1) Cystin according to Hopkins & Cole (l. c.) was looked for but with negative results.

Calculated for $C_{11}H_{12}N_2O_2$ = 64,70% C : 5,88% H.

The substance gave also the Pyrrol reaction, and yielded Indol on fusing with alkali.

II. (a) Precipitate with Phosphotungstic Acid.

The filtrate and washings from the Mercuric Sulphate precipitate were freed from Mercury by H_2S and then precipitated in hot solution with the necessary quantity of Phosphotungstic acid. After standing for 24 hours the precipitate was filtered off, well washed with 3% Phosphotungstic in 5% H_2SO_4 and finally dried by suction. The precipitate suspended in water was decomposed, whilst warm, with Baryta solution then filtered and washed with hot water. During the decompositjon there was a well marked evolution of Ammonia. All free Barium salts exactly removed from the filtrate by means H_2SO_4 and the filtrate concentrated. The further treatment of this filtrate was carried out according to the method of Kossel and Kutscher (15) for the separation of the Hexone bases by means of Silver nitrate.

Owing to the alkalinity of the fluid itself it was found unnecessary to add Baryta for the precipitation of the Histidine fraction. After thorough washing the Histidine silver precipitate was decomposed on the water bath by 10% Hydrochloric acid in slight excess. In order to carry out Kossel and Pattens (16) method, the one which was used for the isolation of the Histidine, is was first necessary to remove the HCl as the Mercuric Sulphate did not give any precipitate in its presence. This removal was carried out by means of silver sulphate and subsequent removal of the excess of Silver by H_2S. A crop of crystals was obtained, which after repeated crystallisation, gave a good Cl figure.

Analysis.

0,1353 grm salt gave 0,1703 AgCl = 31,12% Cl.
Calculated for $C_6H_9N_3O_2$ 2 HCl = 31,14% Cl,

The substance which was obtained on the addition of excess of saturated solution of Baryta to the filtrate from the Histidine fraction, on decomposition of the silver precipitate with H_2S did not give characteristic salts of Arginine. It is being further investigated.

The excess of Baryta having been exactly removed with sulphuric acid and the silver with H_2S, from the filtrate of the Arginine fraction the solution was concentrated and reprecipitated with Phosphotungstic acid, the resulting precipitate being as usual decomposed with Baryta, the excess of Baryta exactly removed and the filtrate concentrated to a syrup and then treated with a saturated alcoholic solution of Picric acid as long a precipitate was formed. The picric salt was filtered, washed well with absolute alcohol, dried, and decomposed with 10% HCl. After complete decomposition the picric acid was removed by concentration and shaking out with Ether. The solution was concentrated to small bulk and allowed to cry-

stallise out. As no Potassium salts could be detected the crystals were dissolved in water, and the solution decolourised with animal charcoal, filtered and concentrated. The crystals which formed were filtered off and recrystallised.

Analysis.

0.1944 g Subst. gave 0.2535 g Ag. Cl = 32.04 % Cl.
Calculated for $C_6H_{14}N_2O_2 2HCl$ = 32,25 % Cl.

Putrescine and Cadaverine were looked for according to the method of Lawrow (17) but without success.

On treating the filtrate from the Lysine picrate according to the method of Kutscher and Lohmann (18) for the detection of choline a small precipitate was obtained with the mercuric chloride but not sufficient for detailed examination.

II (δ). Monamino Fraction.

The filtrate from the precipitation of the Hexone bases with phosphotungstic acid was treated according to E. Fischer's (19) Ester method. The phosphotungstic acid was removed by means of Baryta, excess of Barium exactly removed with H_2SO_4 and the filtrate then concentrated (A sample of this filtrate was tested for aspartic acid by means of silver in neutral solution but with negative result). During the concentration some Tyrosin.

Analysis.

0,1203g Subst. gave by Kjeldahl's method 0,00938g N = 7,79 % N.
Calculated for $C_9H_{11}NO_3$ = 7.75 % N.

along with a little Leucine separated out. The fluid was eventually concentrated to dryness and the whole mass of crystals was then transferred to a large flask with absolute alcohol and esterified as usual. Esterification having been carried out twice and alcohol removed the esters were extracted with ether in the cold. Fractionation took place in a vacuum of from 13—16 mm, with the exception of the first which chiefly consisted of Ether. Resulting fractions were saponified, all those coming over below 90° by means of water alone, those coming over above that temperature by means of freshly crystallised Baryta.

Fraction up to 30°

consisted mostly of Ether.

Fraction 30°—55°

owing to an accident most of this fraction was lost. It yieldad however after saponification and fractional crystallisation amido valerianic acid.

Analysis.

0,0841 g Subst. gave 0,010079 g N = 11,90 % N.
Calculated for $C_5H_{11}NO_2$ = 11,98 % N.

Melting point 281°—282°. E. Fischer 285°—286°.

Fraction 55°—70°.

This fraction amounted to about 55 g. the greatest amount coming over just below 70°. It consisted mainly of Leucine and amido valerianic acid with perhaps some of the lower amido fatty acids. Fractional crystallisation gave a good crop of Leucine crystals.

Analysis.

0.2110 g Subst. gave 0,02212 g N = 10,48 %
Calculated for $C_6H_{13}NO_2$ = 10.70 %

Melting point 298—299°. E. Fischer 293°—295°.

The Copper salt was also obtained.

It was impossible to definitely isolate any of the amido fatty acids lower than amido valerianic acid.

Fraction 70°—80°.

Amounted to about 42 g. This fraction seemed to consist largely of leucine mixed with α-pyrrolidin-carboxylic acid both in its active and racemic forms.

The crystals obtained from this fraction, and from the following fraction (80° 90°) as well, were extracted with boiling absolute alcohol, the alcoholic filtrate concentrated to a syrup, dissolved in water and decolourised with animal charcoal, filtered and again concentrated to a syrup, which was then rapidly extracted with cold absolute alcohol. This alcoholic filtrate was concentrated and resulted in a fair yield of crystals, which were next boiled up with excess of freshly precipitated copper hydroxide. The solution was filtered hot and the filtrate concentrated to dryness on the water bath. The copper salt of the active α-pyrrolidincarbonylic acid was separated from the inactive salt by boiling the dry crystalline mass with absolute alcohol and filtering whilst hot. The copper was removed from the filtrate by means of H_2S, the solution filtered and again concentrated to a syrup. This syrup on treatment with phenylisocyanate yielded typical crystals of the Hydantoin derivative of α-pyrrolidin-carboxylic acid, melting sharply at 142,5, Fischer gives 143°.

The Copper salt insoluble in absolute alcohol was dissolved in water, concentrated, crystallised recrystallised and dried.

Analysis.

0,1118 grm subst. gave 0.00994 grm N = 8.89 % N
Calculated for $C_{10}H_{16}O_4N_2Cu + 2\ H_2O$ = 8,56 % N
0,0706 grm subst. gave 0.0192 grm CuO = 21,73 % Cu
Calculated for $C_{10}H_{16}O_4N_2Cu$ = 21,66 % Cu

Fraction 80°—90°.

This fraction amounted to 32.5 grms and consisted largely of Leucine, contained also some α-pyrrolidincarboxylic acid.

Fraction 90°—125°.

This fraction amounted to 6 grms and was saponified by means of Baryta. The insoluble barium precipitate left on filtering was extremely small. The excess of barium was removed exactly from

the filtrate, by H_2SO_4, which was then concentrated until crystallisation began. A sample was again tested for the presence of aspartic acid by the silver method but with negative result. The more soluble crystalline fraction was dissolved in water, then saturated with dry HCl gas and concentrated on the water bath, where on cooling well formed crystals in rosettes separated out. A sample of these gave a melting point of 194°, Kutscher (20) gaves 193° as the melting point of glutamic acid hydochloride. Further analyses are given with the next fraction. A little Leucine was also present in this fraction.

Fraction 125°—170°.

This amounted to about 11.5 grm. This fraction had added about twice its volume of Ether, and was then shaken up in a separating funnel, three times, with double the amount of water, in order to separate Phenylalanine from aspartic and glutamic acids if present, these latter going into the watery solution. *a*) The watery solution was now saponified by means of Baryta. The insoluble baryta compound left was very small. The excess of Barium in filtrate which was extremely fluorescent, was exactly removed by H_2SO_4. The filtrates were concentrated, decolourised with animal charcoal, filtered and evaporated to dryness. In order to remove any Pyrrolidoncarboxylic present crystals mass was boiled up with absolute alcohol as advised by Fischer (21). The alcoholic extract again decolourised with animal charcoal and concentrated to a thick syrup, which however shewed no tendency to crystallise. The crystals insoluble in alcohol were dissolved up in water, and the solution concentrated. The crystals so obtained were recrystallised from water and dried.

Analysis.

0,1925 grm Subst. gave 0,2889 grm CO_2 0,1042 grm H_2O.
Found = 40,93% C: 6,05% H.
Calculated for $C_5H_9NO_4$ = 40,81% C: 6,12% H.
0,1022 grm Subst. gave 0,0098 grm N = 9,58% N.
Calculated for $C_5H_9NO_4$ = 9,54% N.
Melting point 199°—200°, E. Fischer 204°.

B. The Ether extract of this fraction had the ether removed by evaporation a deep brown oily substance being left. This when treated with HCl to convert any Phenylalanine present into the Hydrochloride gave a few crystals of some substance which could not however be completely separated from the oily mass. On treating a little of it with Potassium Bichromate and Sulphuric acid and heating, a hyacinth like odour was emitted. The oily substance gave a marked Millon reaction and a white precipitate with Bromine water in a small sample dissolved in hot water.

Conclusions.

1. The following substances have been definitely found in the products of digestion of fibrin with the enzyme obtained from the urine: Histidine, Lysine, Tyrosine, Leucine, Amidovalerianic acid, *α*-pyrro-

lidincarboxylic acid. Glutamic acid, Tryptophane, Ammonia, and undigested proteid-albumoses etc., and in addition Arginine, Phenylalanine and Alanine were also probably present.

2. No Aspartic acid was found. Xanthine and Pyrimidine bases were also looked for but unsuccessfully.

3. There exists in normal urine a ferment or ferments capable of acting in an alkaline medium and giving rise to products after prolonged digestion similar to those formed by Trypsine under similar circumstances.

The presence of Tryptophane after such prolonged digestion is interesting, and may be taken as a priori evidence that the digestion was carried out under approximately aseptic conditions.

In conclusion I wish to offer my very best thanks to Dr. Hedin for much kindness and advice during the course of this research.

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ON THE ANTITRYPTIC ACTION OF NORMAL SERUM.

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The power that normal serum possesses of hindering or preventing the action of trypsin was, so far as I have been able to find, first definitely noticed by Hahn [1], though it was pointed out almost contemporaneously by Pugliese and Coggi [2]. Several years earlier however Fermi and Pernossi [3] had noticed that trypsin rapidly disappeared after injection into the animal body, and that it was destroyed by contact with the tissues *in vitro*. Since then the question has been investigated by Achalme [4], Camus and Gley [5], Charrin and Levaditi [6], Simnitzki [7], and Delezenune [8], but in rather a cursory fashion. In two papers of recent origin by Landsteiner [9] and Glaessner [10] attempts were made to isolate that fraction of the serum to which the antibody is attached. The results obtained by these two investigators did not however agree.

Landsteiner concluded that the anti-action was intimately connected with the albumin fraction, *i.e.* the fraction coming down between half and full saturation of the serum with ammonium sulphate. Glaessner, on the other hand, came to the conclusion that it was associated with the euglobulin, *i.e.* the fraction precipitated by $\frac{1}{3}$ saturation with am. sulph. Both observers used Mett's tubes as the means of gauging the degree of digestion.

Methods. The method of fractionating the serum which I employed was essentially that described by Freund and Joachim [11]. The serum was obtained from various species of animals, but usually from the ox. The blood was allowed to stand in a cool place over night, the serum which had separated was pipetted off and freed by means of the centrifuge from corpuscles. A measured quantity of this serum was diluted with two or three times its volume of distilled water, and cold saturated solution of ammonium sulphate added up to $\frac{1}{3}$ saturation. After the removal of the euglobulin by filtration the concentration of the filtrate was brought up to $\frac{1}{2}$ saturation by the further addition

of ammonium sulphate solution. The pseudoglobulin was filtered off and the filtrate fully saturated by the addition of the requisite amount of solid dry ammonium sulphate, which threw down the albumin fraction. Both the euglobulin and pseudoglobulin were purified by several reprecipitations. The final precipitates of all three fractions were dissolved or suspended in as little water as possible and transferred to parchment dialysers, and dialysed against running water, in some instances against distilled water, to remove the ammonium sulphate. The contents of the dialysers were then brought up to the original volume of the serum employed, either by means of distilled water or by 0·25 % sodium carbonate solution.

The trypsin solutions were obtained by digestions of ox or other pancreases. The digestion was carried on until practically no biuret reaction was given, the fluid was then dialysed to get rid of most of the crystalline products, so that the N. figure of this solution might be as low as possible.

In the majority of cases, particularly during my earlier experiments, the substance used as material for digestion was coagulated blood serum. In order to get accurate amounts of this, the fluid serum was pipetted off in the required amount into suitable digestion bottles, which were then placed for half-an-hour in a water-bath heated to boiling point. The resulting coagulum was next cut up, still of course in the bottle, into cubes of as near one size as possible. In the later series, however, the material employed was a 3½ % solution of caseinogen in 0·25 % sod. carb. solution. This formed a very suitable medium, especially for quantitative work, as it shows very small differences in the degree of digestion.

The mode of analysis employed was that used by Hedin[12] in his work on the spleen enzymes, viz. precipitation by means of a solution of tannic acid[1], and the estimation of the N. in the filtrate was made by Kjeldahl's method. In the following tables of results the figures given are the number of c.c. of $\frac{N}{10}$ H_2SO_4 required to neutralise the ammonia distilled over. The tannic acid solution was chosen as being the easiest to use. It precipitates all substances higher than peptones,

[1] Tannic acid solution:

Tannic acid	100 grms.
Sodium acetate	25 ,,
Sodium chloride	75 ,,
Glacial acetic acid	50 c.c.
Distilled water	to 1000 ,,

and to my mind it gives more accurate results than can be obtained by the use of Mett's tubes.

That there exists such a body as antitrypsin the figures (see Table I) show. Not only do they demonstrate the fact that serum itself possesses this anti-action, but they go further and support Landsteiner as against Glaessner in his contention that the anti-action is in connection with the so-called albumin fraction. In this series for purposes of comparison I have demonstrated, (1) the use of boiled fractions ($\frac{1}{2}$ hour at 100°), and (2) the results of using boiled trypsin solution ($\frac{1}{2}$ hour at 100°). In the latter case it may be noted as a matter of some interest, that the euglobulin fraction shows a little more digestion than either of the other two. This may be taken as demonstrating the presence of the proteolytic enzyme found by Hedin[13] in the blood of the ox and attached to the euglobulin fraction. It may be also worthy of note that in a number of instances one finds that the pseudoglobulin fraction possesses some slight antitryptic action, which is probably to be attributed to the fraction not having been completely freed from albumin.

TABLE I. *Precipitate with Tannic Acid*[1]. *5 c.c. of filtrate for analysis.*

	Medium		Fraction		Trypsin	$\frac{N}{10}H_2SO_4$
A.	Control. (Pptd. immediately.)					
	5 c.c. Coag. Serum +	5 c.c.	Euglobulin sol.	+	5 c.c.	0·4 c.c.
	,, ,,	,,	Pseudoglob. ,,		,,	0·4 ,,
	,, ,,	,,	Albumin ,,		,,	0·2 ,,
	,, ,,	,,	Serum ,,		,,	0·5 ,,
B.	Digest. 4 days at 37°.					
	5 c.c. Coag. Serum +	5 c.c.	Euglobulin sol.	+	5 c.c.	6·8 c.c.
	,, ,,	,,	Pseudoglob. ,,		,,	6·9 ,,
	,, ,,	,,	Albumin ,,		,,	0·9 ,,
	,, ,,	,,	Serum ,,		,,	1·1 ,,
C.	5 c.c. Coag. Serum +	5 c.c.	Euglobulin sol. boiled	+	5 c.c.	7·9 c.c.
	,, ,,	,,	Pseudoglob. ,, ,,		,,	7·3 ,,
	,, ,,	,,	Albumin ,, ,,		,,	7·2 ,,
D.	5 c.c. Coag. Serum +	5 c.c.	Euglobulin sol.	+	5 c.c. boiled	1·2 c.c.
	,, ,,	,,	Pseudoglob. ,,		,, ,,	0·5 ,,
	,, ,,	,,	Albumin ,,		,, ,,	0·35 ,,

[1] Of course all digests of series are precipitated with the same amount of acid. In all series given in this research 10 c.c. was the amount of tannic acid solution used.

This anti-action is quite as pronounced when solutions of caseinogen or boiled antibody solutions are used in place of the coagulated serum (see Table II). I also tried coagulated egg albumen, but this digested too slowly owing to the marked antitryptic action, as Vernon[14] recently pointed out, which this proteid possesses.

TABLE II. *Precipitate with Tannic Acid.* 10 *c.c. of filtrate for analysis.*

	Antibody	Digest Substance	Trypsin	0·25 % Na_2CO_3	Before digestion $\frac{N}{10}$ H_2SO_4	After 20 hours' digestion at 37° $\frac{N}{10}$ H_2SO_4
A.	5 c.c. Serum Albumin	10 c.c. $3\frac{1}{2}$ % Casein sol.	5 c.c.	——	0·8 c.c.	2·0 c.c.
	———	,, ,,	,,	5 c.c.	0·5	11·9
B.	5 c.c. Serum Albumin	10 c.c. boiled Serum Alb.	,,	——	0·5	0·6
	———	,, ,,	,,	5 c.c.	0·4	3·0
C.	5 c.c. Serum Albumin	———	,,	5 ,,	0·25	0·35

The objection might be raised that the above anti-action is more apparent than real, and that digestion actually occurred but did not go so far as to be detected by the tannic acid method of precipitation which was employed. That such was not the case was shown by a series of experiments where visual examination was the mode employed for observing the degree of digestion.

A variation (see Table III) of this experiment was made where cubes of egg albumen, of approximately equal size, were stained as homogeneously as possible with carmine. After the excess of the stain had been removed by thorough washing, weighed amounts of the cubes were placed in mixtures of the trypsin and the various fractions as usual. The amount of stain set free after digestion of about a week's duration was estimated much after the method employed for hæmoglobin. The solution which I employed for diluting was 20 % acetic acid, as I found that this acid gave the brightest and sharpest colour. In the first place to 1 c.c. of the filtrate from the digests, in test-tubes of very small diameter, was added 1 c.c. of the acid. The figures in Table III give the further additions of acid necessary before there was uniformity in colour.

TABLE III. *Digested at* 37° *for* 7 *days.*

					Trypsin	No. of c.c. Acid added
$2\frac{1}{2}$ grms.	Coag.	Egg	Albumin +	5 c.c. Euglobulin sol. +	5 c.c.	4 c.c.
,,	,,	,,	,,	Pseudoglob. ,,	,,	3
,,	,,	,,	,,	Albumin ,,	,,	1
,,	,,	,,	,,	0·25 % Na_2CO_3	,,	$3\frac{1}{2}$

This result is at best a very crude one, as it must be remembered that a certain amount of diffusion would take place, but it bears out very satisfactorily the more elaborate tannic acid estimations.

Experiments with trypsins and sera of other animals, horse, pig, etc. gave identical results.

I have attempted to ascertain if this reaction is absolutely specific, that is, "Does anti-action only occur when one uses serum and trypsin from the same animal or species?" Glaessner in his paper answers the question in the affirmative, but his figures point not to a real specificity but to one of degree only, *i.e.* the serum of one animal hindered but did not completely prevent the action of the trypsin of an animal of another species. The results which I have obtained indicate that the action is not specific in an *absolute* sense. As to whether *partial* specific action exists it is impossible, with the present methods, to say, since, so far, I have not been able to standardise either the trypsin or antibody solutions. For instance in the experiments detailed in Table IV the ox serum holds the ox trypsin in check, as it likewise does the pig trypsin; but on the other hand pig serum does not inhibit so completely the action of ox trypsin as it does that of pig trypsin. In this result however there must be taken into account the fact that the ox trypsin used was much more active than the pig trypsin. This is shown by the results of digestion (see Table) in the absence of the antibody. In fact if one deducts the figures denoting the degree of digestion in the samples containing antibody from those where the antibody is absent one finds that the inhibition in the case of the pig serum is almost the same as with ox serum when ox trypsin is used, and with pig trypsin the figures obtained are almost identical.

TABLE IV. *Precipitate with Tannic Acid.* 5 *c.c. of filtrate for analysis.*

Medium	Serum	Trypsin	Before digestion $\frac{N}{10}$ H_2SO_4	After 3 days' digestion $\frac{N}{10}$ H_2SO_4	Digestion with Pig's Trypsin without Antibody $\frac{N}{10}$ H_2SO_4	Digestion with Ox Trypsin without Antibody $\frac{N}{10}$ H_2SO_4
5 c.c. Coag. Serum	5 c.c. Ox	5 c.c. Ox	0·8 c.c.	0·75 c.c.	——	8·2 c.c.
,, ,,	,, Ox	,, Pig	0·5	0·7	5·7 c.c.	——
,, ,,	,, Pig	,, Ox	0·5	1·4	——	8·2
,, ,,	,, Pig	,, Pig	0·4	0·6	5·7	——

The view that the action is not absolutely specific is quite in accordance with that reached by Mesnil[15]. Of course one may remember that Morgenroth[16] among others has found that an anti-substance can be produced (by artificial injection) against a vegetable

rennet—cyanarase—which is practically inactive when tested with rennet of animal origin. The experiments of Gessard[17] with tyrosinase of animal and vegetable origin gave results similar to Morgenroth's.

In view of the insistence with which Glaessner maintains that antitrypsin is attached to the euglobulin fraction I made some further experiments to see whether there might not be two antibodies for trypsin present in the serum, similar to the true and pseudo-antibody for rennet found by Korschun[18] in horse's serum. To this end I prepared a solution of the globulins, obtained by the addition of a very small quantity of acetic acid to serum which had been diluted with several times its volume of water. The resulting precipitate was carefully collected and thoroughly well washed with water. It was then dissolved in a small quantity of 0·25% sod. carb. solution (globulins from about 300 c.c. serum dissolved in 100 c.c. sod. carb. sol.). Digestion experiments were performed (see Table V), (1) with and without casein and globulins; (2) with globulins heated to 70° for ½ hour (in order to destroy the proteolytic enzyme normally present in the serum (Hedin)); and (3) with globulins which had been heated to 100° for half-an-hour with and without casein.

TABLE V. *Precipitate immersed with Tannic Acid.* 10 *c.c. of filtrate for analysis.*

					Trypsin	$\frac{N}{10}H_2SO_4$
A.	Control.					
	5 c.c. Globulin sol.	+	5 c.c. 0·25 % Na_2CO_3	+	1 c.c.	0·3 c.c.
	,, ,, ,,		5 c.c. sat. sol. Casein		,,	0·4 ,,
	5 c.c. sat. sol. Casein		5 c.c. 0·25 % Na_2CO_3		,,	0·35 ,,
B.	Digest. 4 days at 37°.					
	5 c.c. Globulin sol.	+	5 c.c. 0·25 % Na_2CO_3	+	1 c.c.	0·7 c.c.
	,, ,, ,,		5 c.c. sat. sol. Casein		,,	5·4 ,,
	5 c.c. sat. sol. Casein		5 c.c. 0·25 % Na_2CO_3		,,	5·0 ,,
	5 c.c. Globulin sol. heated ½ hour at 70°	+	,, ,, ,,	+	,,	2·8 ,,
	5 c.c. Globulin sol. heated ½ hour at 70°		5 c.c. sat. sol. Casein		,,	6·4 ,,
	5 c.c. Globulin sol. heated ½ hour at 100°	+	5 c.c. 0·25 % Na_2CO_3	+	,,	2·2 ,,
	5 c.c. Globulin sol. heated ½ hour at 100°		5 c.c. sat. sol. Casein		,,	6·3 ,,

This table demonstrates the fact that there is no antibody to be detected, at least by the methods which I have employed, in the (eu)globulin fraction. The amount of digestion of casein alone is

represented by 5 c.c. of $\frac{N}{10}$ H_2SO_4, whilst when both casein and globulin solution are present together it equals 5·4 c.c. in the unheated and 6·4 c.c. $\frac{N}{10}$ H_2SO_4 with globulins heated to 70°.

This table together with the experiments in Table I bear out Oppenheimer and Aron's[19] statement that the globulins in their "native" state, *i.e.* unheated normal state, are difficult to attack by means of trypsin, and suggest that the globulins only are not acted on by trypsin.

With regard to the temperature at which the antibody is destroyed, it seems to depend to some extent on whether one is dealing with the isolated antibody, *i.e.* the serum albumin fraction, or with the whole unheated serum, and also whether distilled water or 0·25 % sod. carb. solution is used to dissolve the antibody. The presence of the alkali, as my experiments show, exercises rather a harmful influence, in so far that it causes the anti-action to be suspended at a lower temperature than is the case where the alkali is absent. This deleterious action of the sod. carb. is quite as marked as when serum itself is employed in the experiment. Madsen in his experiments with antirennin likewise found the presence of alkali to have a harmful effect.

Table VI. illustrates the effect of heat upon the isolated serum albumin containing the antibody, at the same time demonstrating the destructive action of the alkali when this is present. In each case the serum albumin was kept in the water-bath at the given temperature for half-an-hour.

TABLE VI. *Precipitate with Tannic Acid.* 10 *c.c. of filtrate for analysis.*

A. Control. (Pptd. immediately.)

Medium	Serum Albumin	Water	Trypsin	$\frac{N}{10}$ H_2SO_4
5 c.c. Coag. Serum	5 c.c. Active	5 c.c.	5 c.c.	0·5 c.c.

B. Digest. 4 days at 37°.

Medium	Serum Albumin	Trypsin	Digest + 5 c.c. H_2O $\frac{N}{10}$ H_2SO_4	Digest + 5 c.c. 0·25 % Na_2CO_3 $\frac{N}{10}$ H_2SO_4
5 c.c. Coag. Serum	5 c c. Active (37°)	5 c.c.	0·85 c.c.	0·8 c.c.
,, ,,	,, 45°	,,	0·8	0·75
,, ,,	,, 50°	,,	0·85	0·8
,, ,,	,, 55°	,,	1·2	4·05
,, ,,	,, 60°	,,	11·0	13·5
,, ,,	,, 100	,,	12·75	13·4

In the case of serum itself the same difference is manifest (see Table VII). The various digests were kept in the water-bath at the temperature given for half-an-hour.

TABLE VII. *Precipitate with Tannic Acid.* 10 *c.c. of filtrate for analysis.*

Medium	Serum		Trypsin	Digest + 5 c.c. H_2O $\frac{N}{10}H_2SO_4$	Digest + 5 c.c. 0·25 % Na_2CO_3 $\frac{N}{10}H_2SO_4$
A. Control. (Pptd. immediately.)					
5 c.c. Coag. Serum	5 c.c. Active		5 c.c.	1·1 c.c.	0·9 c.c.
B. Digest. 2½ days at 37°.					
5 c.c. Coag. Serum	5 c.c. Active	(37°)	5 c.c.	1·7 c.c.	0·95 c.c.
,, ,,	,,	55	,,	1·65 ,,	0·9 ,,
,, ,,	,,	60°	,,	1·6 ,,	8·2 ,,
,, ,,	,,	65°	,,	1·7 ,,	16·1 ,,
,, ,,	,,	70°	,,	2·0 ,,	18·1 ,,
,, ,,	,,	100°	,,	15·5 ,,	20·5 ,,

The experiments show that the antibody is destroyed by heating for half-an-hour, in the presence of 0·25 % sod. carb., at a temperature between 60° and 65° in the case of the "whole" serum, and between 55° and 60° for the isolated fraction. When water is used the temperature in the case of the isolated fraction is apparently much about the same, viz. 55° to 60°, but for the serum itself it is higher, viz. about 70°. These figures agree very well with those of Hahn (*l.c.*) and Delezenne (*l.c.*) obtained when working with "whole" serum.

As to the effect of room temperature (about 15°) on the antitrypsin it was found that solutions of the antibody retain at this temperature a marked degree of anti-action for lengthy periods, both when in isolated form and in serum. When kept in the cold room (5°) the rate of destruction progresses, as would be expected, much more slowly; even after several months active samples can be easily obtained.

The results which Morgenroth obtained when working with anti-rennin differed markedly from the above, as he found that this antibody deteriorated rapidly, even when kept in the cold room.

The effect of drying has likewise been tried (see Table VIII). The antibody precipitated by ammon. sulph. was allowed to dry on a filter-paper at room temperature, the presence of the Am_2SO_4 preventing putrefaction. After the mass was completely dried it was scraped off the filter-paper and finely powdered. A portion of this powder was dissolved in water and transferred to a dialyser in order to get rid of the sulphate. When the solution was free from the salt digests were prepared.

TABLE VIII. *Precipitate with Tannic Acid.* 10 *c.c. filtrate for analysis.*

	Serum Albumin		Trypsin		$\frac{N}{10}$ H_2SO_4
A. Control. (Pptd. immediately.)					
5 c.c. Coag. Serum	+ 5 c.c. Dialysed (dry)	+	5 c.c.	–	0·7 c.c.
B. Digest. 4 days at 37°.					
5 c.c. Coag. Serum	+ 5 c.c. Dialysed (dry)	+	5 c.c.	=	1·1 c.c.
,, ,,	+ ,, ,, heated ½ hour at 100°	+	,,	=	6·8

These figures show that the property of hindering the action of the enzyme had been retained by the dry albumin.

Dialysis has not been found during the course of this research to cause much diminution of the activity of the solution of the antibody. It was only on very rare occasions that a serum which possessed anti-action was found when fractionated and dialysed to be inert. Such cases have occurred once or twice, but the cause remained undiscovered, as the methods were those usually attended by excellent results.

Delezenne (*l.c.*) maintains that this anti-action is not an antitryptic one, but that it is an antienterokinase (antikinasique). Quite recently Ascoli and Bezzola[20] took up this question and concluded that the antibody acted in both ways. Whatever may have been the nature of the solutions of trypsin and antibody which Delezenne worked with, the antibody I had was really antitryptic, as my solutions of trypsin contained no trypsinogen, for on the addition of a solution of enterokinase there was no increase in their activity. I do not maintain that antienterokinase does not exist, in fact all evidence rather points to the occurrence of such a body.

In conclusion I wish to offer my best thanks to Dr Hedin for much kindness and advice during the carrying out of this piece of work.

CONCLUSIONS.

1. This anti-action of normal serum against trypsin is found in connection with the so-called albumin fraction, *i.e.* the fraction precipitated between half and full saturation with ammonium sulphate. This result agrees with that of Landsteiner.

2. Globulins do not possess antitryptic action, but are only very slowly attacked by the enzyme (cf. Oppenheimer and Aron).

3. This anti-action, as Glaessner also noted, was found in all varieties of sera examined.

4. The anti-action is effective with all varieties of proteid, whether in solid or fluid form.

5. Absolute specificity does not exist, and existence of partial specificity is questionable.

6. The isolated antibody—the albumin fraction—is rapidly injured by heating. A temperature of 55° for half-an-hour suffices both in presence and absence of alkali to destroy to a large extent the anti-action.

7. Anti-action of serum itself is somewhat more resistant. Ill effects noted by heating for half-an-hour at 60° in presence of alkali and about 70° in its absence.

8. Solutions of antibody do not deteriorate rapidly even at room temperature.

9. Dried antibody retains its anti-ferment action very well.

10. Dialysis, as a rule, has no apparent destructive influence.

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DEPARTMENT OF PATHOLOGY

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[From THE JOURNAL OF HYGIENE, Vol. V, No. 2, April, 1905.]

THE SEASONAL PREVALENCE OF HOFMANN'S BACILLUS[1].

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(*From the Lister Institute of Preventive Medicine.*)

During the last six years (1899—1904) some fifteen thousand examinations of material from throats for the diphtheria bacillus have been made at the Lister Institute. The localities from which this material has been derived have naturally been widespread and varied; much of the work however has been done for local authorities in and near London, and the sources of origin of the major part of the material have not varied very much during the whole period under review.

Records have been kept throughout of the occurrence both of the genuine Klebs-Loeffler organism and of the pseudo-diphtheria bacillus of Hofmann. The diagnosis is based upon the microscopical appearances found in young (12—20 hours) cultures on serum made from the swabs sent to the Institute. The films are made from smears taken over the whole surface of the culture and no attempt is made to pick out individual colonies. These preparations have been made by the same laboratory attendant during the whole period dealt with here. In the ordinary routine, Loeffler's blue is alone used, though a certain number are also examined by Neisser's method. From time to time the accuracy of the diagnoses has been tested in a few instances by isolation of the organisms and investigation of the cultural and pathogenic pro-

[1] I have not attempted any survey of the literature of the subject: this has been recently reviewed in the exhaustive papers of G. S. Graham-Smith; this *Journal*, vol. III. p. 216, vol. IV. p. 258.

TABLE I.

	A		B		C		D	
	Klebs-Loeffler per thousand of total cases examined	Percentage above (+) or below (−) mean (282)	Klebs-Loeffler per thousand of cases without Hofmann	Percentage above (+) or below (−) mean (306)	Hofmann per thousand of total cases examined	Percentage above (+) or below (−) mean (111)	Hofmann per thousand of cases without Klebs-Loeffler	Percentage above (+) or below (−) mean (142)
January	264	− 6·4	285	− 6·9	**115**	**+ 3·6**	139	− 2·1
February	249	− 11·7	268	− 12·4	87	− 21·6	111	− 21·7
March	265	− 6·0	292	− 4·6	**118**	**+ 6·3**	**151**	**+ 6·3**
April	269	− 4·6	289	− 5·5	105	− 5·4	131	− 7·7
May	**283**	**+ 0·3**	**308**	**+ 0·6**	**126**	**+13·5**	**157**	**+10·6**
June	273	− 3·2	303	− 1·0	**128**	**+15·3**	**164**	**+15·5**
July	282	± 0·0	**307**	**+ 0·3**	**123**	**+10·8**	**154**	**+ 8·4**
August	280	− 0·7	**311**	**+ 1·6**	**144**	**+29·7**	**181**	**+27·5**
September	**289**	**+ 2·5**	**313**	**+ 2·3**	101	− 9·0	132	− 7·0
October	**321**	**+13·8**	**345**	**+12·7**	104	− 6·3	136	− 4·2
November	**303**	**+ 7·4**	**333**	**+ 8·8**	104	− 6·3	**143**	**+ 0·7**
December	273	− 3·2	290	− 5·2	88	− 20·7	110	− 22·5
Average for whole period of 72 months	282		306		111		142	

The monthly percentages which are above the mean are printed in heavy type.

perties of pure cultures. Though the necessities of time and purpose have presumably led to a certain number of mistakes, the differential diagnosis between the Klebs-Loeffler and Hofmann organisms can in general be readily made by the simple method which has been used. The morphological differences are usually obvious enough, especially when taken in conjunction with the tinctorial differentiation brought out by Loeffler's alkaline methylene blue. Strictly speaking, however, the records refer to the presence of "bacilli morphologically indistinguishable from" the Klebs-Loeffler and Hofmann organisms respectively. The actual diagnoses have been made by Prof. R. T. Hewlett, Dr Sidney Rowland, Dr A. T. MacConkey, Dr A. Moore, and a few by myself, in conjunction with Dr Allan Macfadyen.

As far as possible I have eliminated from the records those cases in which swabs were taken from persons who were merely "contacts" of diphtheria infections. The remainder comprise those who were suffering either from true diphtheria or from some affection of the throat bearing a likeness to diphtheria sufficiently close to render the bacteriological examination desirable. They number in all 14937; of these

4069 or 272 per thousand showed Klebs-Loeffler alone,

1521 or 102 per thousand showed Hofmann alone,

139 or 9 per thousand showed Klebs-Loeffler and Hofmann together.

So that, in all, Klebs-Loeffler was present in 282 per thousand and Hofmann in 111 per thousand of all cases examined.

In order to investigate the question of seasonal prevalence, the proportion of examinations in which each organism was found has been calculated for each month. The details are given in the Appendix; the summarised totals are shown in Table I, and represented graphically in Fig. 1.

These show a clear difference in the seasonal variations of the frequency of positive examinations for the two organisms, *Klebs-Loeffler prevailing during September, October and November, while Hofmann is most frequent from May to August.* The curve of frequency of finding Klebs-Loeffler corresponds fairly closely with the well-known seasonal curve for the occurrence of cases of diphtheria. This points to the conclusion that the actual seasonal prevalence of Hofmann is similar to that shown in the curve of frequency of finding that bacillus in the swabs examined.

In the present series, Hofmann is much less frequently found in

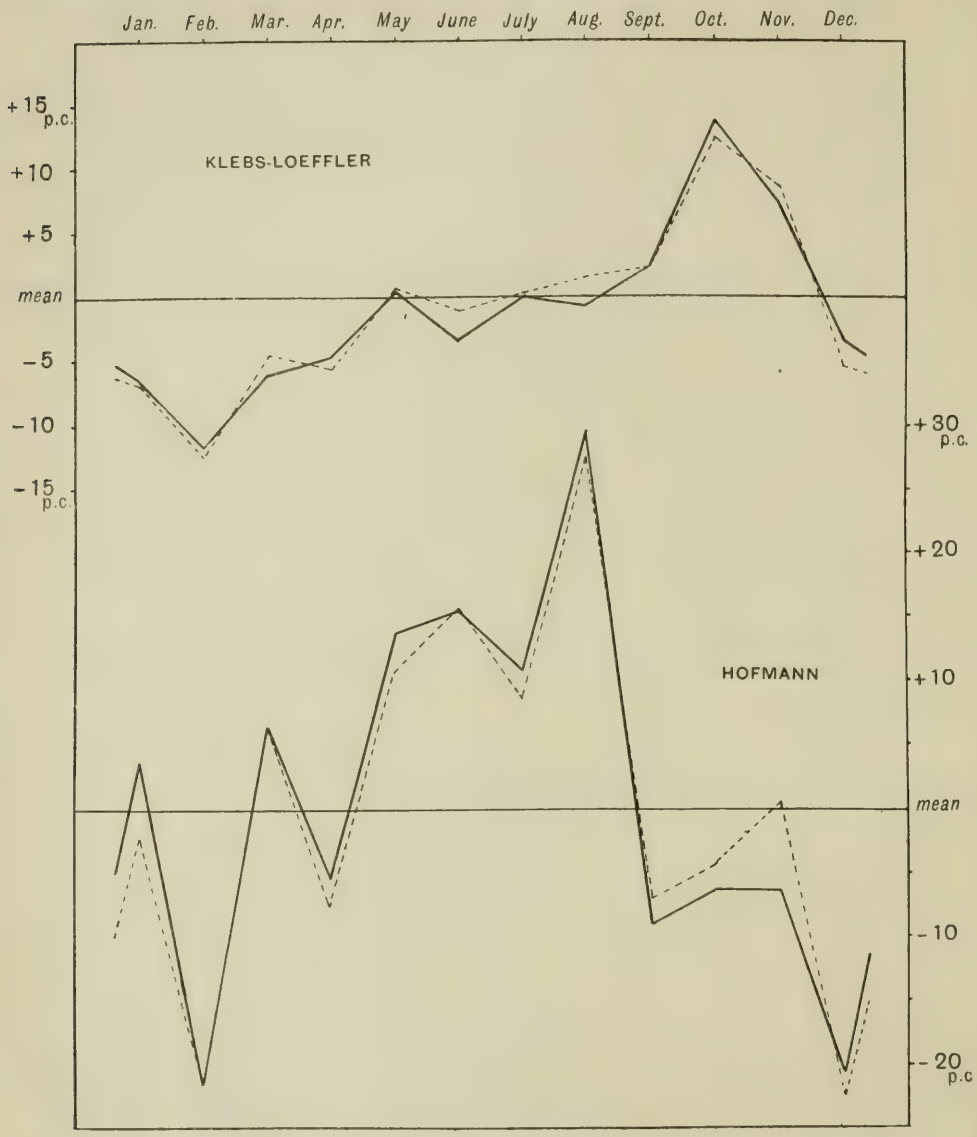

Fig. 1. The abscissae represent months; the ordinates the percentage of the mean for the whole period by which the percentage of positive examinations in each group deviates above or below the mean. The upper curves represent the proportion of Klebs-Loeffler found (1) in all cases examined (Table I, A) by the continuous line, and (2) in cases without Hofmann (Tables I, B) by the broken line.

The lower curves represent the proportion of Hofmann found (1) in all cases examined (Table I, C) by the continuous line, and (2) in cases without Klebs-Loeffler (Table I, D) by the broken line.

cases with, than in those without Klebs-Loeffler[1]. Hence any rise in the Klebs-Loeffler curve would tend to automatically lower the Hofmann curve over the same period, if both curves are based on the percentage frequency of positive examinations in all cases examined. That this factor is immaterial in the present instance is shown by the fact that the percentage deviations of the percentages of positive results for Klebs-Loeffler in cases without Hofmann, and for Hofmann in cases without Klebs-Loeffler (Figures 1 and 2, dotted curves) correspond with those for the same organism in all cases examined.

Corresponding figures for each year are given in Table II and shown graphically in Fig. 2.

As far as the percentage of all examinations which show Klebs-Loeffler is concerned, there has been a considerable fall from 1899 (336 per thousand positive) to 1903 (180 per thousand positive) with a slight recovery in 1904. There has been no corresponding decline in the number of cases of diphtheria notified in London or in England generally during the same period. The explanation would appear to be that there has been an increasing tendency to call in the aid of bacteriology on slighter grounds, and to send swabs for examination from cases which have a more remote clinical resemblance to diphtheria.

The yearly figures for Hofmann give a curve which resembles the Klebs-Loeffler curve, though the fall has been greater throughout. If the explanation given above of the fall in the Klebs-Loeffler curve is correct, the natural conclusion to draw from the similar decline in the Hofmann curve is that Hofmann's bacillus is associated with some morbid condition of the throat which resembles, but is not identical

[1] Hofmann was found in 33 per thousand of cases with, and in 142 per thousand of cases without, Klebs-Loeffler. It is probable that these figures by no means represent, at any rate quantitatively, the real frequency of co-existence. In the first place, once Klebs-Loeffler has been found in the film, further search is not always made for Hofmann. Secondly, and perhaps most cogently, if the swab is taken accurately from a definite membrane, Klebs-Loeffler may be obtained in pure culture as being the causative organism; if Hofmann has no relation to the local disease, it would probably be absent from the acute specific local lesion. It would be interesting to know how often under these circumstances it is present in other areas of the mouth, nose and pharynx. In the third place, the possibility of the overgrowth of Hofmann by Klebs-Loeffler on a medium favourable to the latter must be considered; this does not however seem to take place in artificial mixtures grown on serum.

It may be not without significance that both organisms have been found more frequently together in monthly and yearly periods which correspond more closely with the prevalence of Hofmann than with that of Klebs-Loeffler (see Appendix, Tables G and H). The cases are however very few in number.

TABLE II.

	A		B		C		D	
	Klebs-Loeffler per thousand of total cases examined	Percentage above (+) or below (−) mean (282)	Klebs-Loeffler per thousand of cases without Hofmann	Percentage above (+) or below (−) mean (306)	Hofmann per thousand of total cases examined	Percentage above (+) or below (−) mean (111)	Hofmann per thousand of cases without Klebs-Loeffler	Percentage above (+) or below (−) mean (142)
1899	**336**	**+19·1**	**377**	**+23·2**	**163**	**+46·8**	**215**	**+51·4**
1900	**316**	**+12·1**	**353**	**+15·4**	**152**	**+36·9**	**197**	**+38·7**
1901	**324**	**+14·9**	**350**	**+14·4**	99	−10·8	139	− 9·1
1902	269	− 4·6	295	− 3·6	104	− 6·3	137	− 3·5
1903	180	−36·2	191	−37·6	67	−39·6	79	−44·4
1904	231	−18·1	242	−20·9	62	−44·1	75	−47·2
Average for whole period	282		306		111		142	

The yearly percentages which are above the mean are printed in heavy type.

[illegible]heria, and that, with a more extended use of the bacterio-[illegible] test, the examples of this condition have, like those of true [illegible]heria, been numerically diluted by an increasing proportion of relatively normal cases. If Hofmann's bacillus were a common inhabitant of the throat, giving rise to no pathological changes, it would be natural to

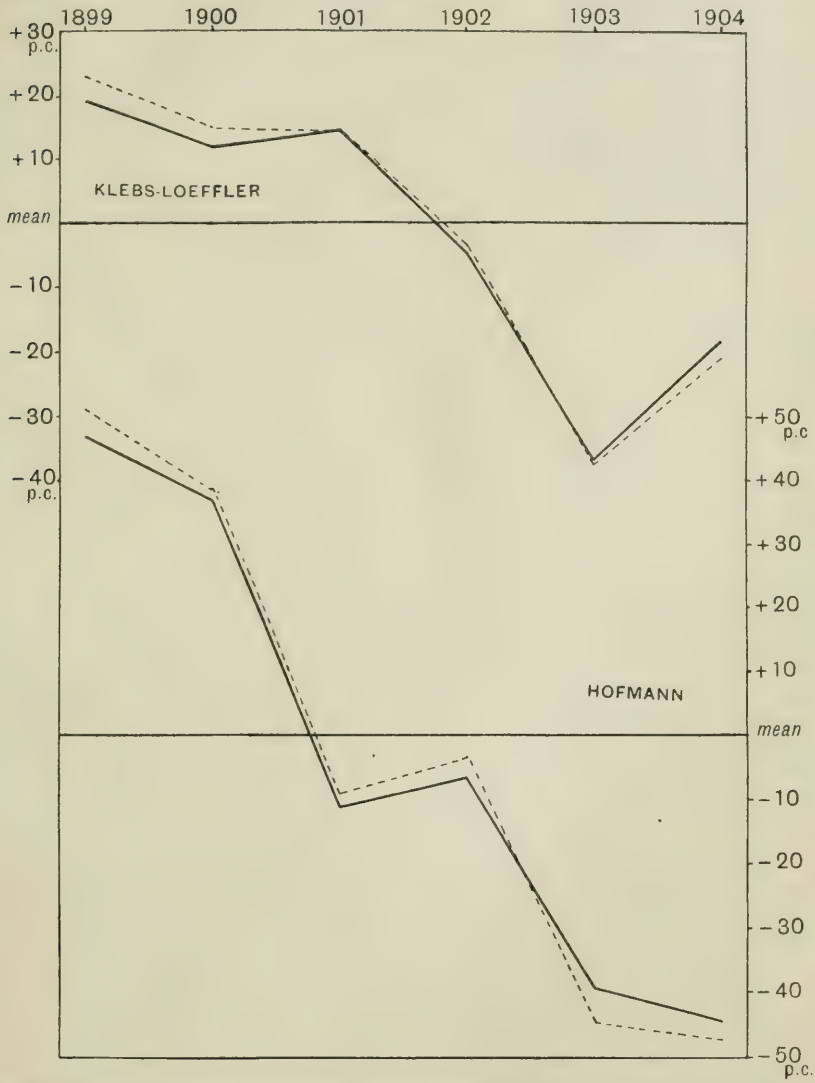

Fig. 2. The abscissae represent years. Otherwise the curves are constructed precisely as in Fig. 1, except that the vertical scale is diminished by one-half.

TABLE E. *Cases per thousand of total cases examined in wh was found (including Klebs-Loeffler with Hofmu.*

Year	Jan.	Feb.	March	April	May	June	July	August	Sept.	Oct.	Nov.	Dec.	Total
1899	**399**	314	312	303	**348**	287	**352**	271	**402**	314	**387**	325	336
1900	313	**337**	**399**	247	**349**	269	272	**362**	**322**	**402**	263	243	316
1901	320	241	251	**333**	268	299	296	256	**347**	**366**	**365**	**349**	324
1902	219	233	**288**	**300**	**343**	**286**	**298**	**280**	180	**294**	**275**	246	269
1903	**181**	133	156	**288**	**225**	**206**	**240**	**270**	131	113	117	144	180
1904	167	**278**	177	134	117	**297**	202	217	180	**341**	**317**	**253**	231
Total	264	249	265	269	283	273	282	280	289	321	303	273	282

Percentages which are above the mean for each year are printed in heavy type.

TABLE F. *Cases per thousand of total cases examined in which Hofmann was found (including Klebs-Loeffler with Hofmann).*

1899	**187**	**180**	123	**177**	**228**	**191**	**178**	**165**	131	133	129	**165**	163
1900	**244**	144	**159**	131	**165**	**183**	134	128	128	140	**164**	57	152
1901	85	24	**138**	90	84	86	**120**	**244**	**130**	**109**	77	48	99
1902	61	75	87	75	**149**	**136**	**149**	**127**	83	**112**	104	**111**	104
1903	**82**	48	52	**81**	**79**	**109**	**89**	**116**	48	38	39	26	67
1904	36	40	**95**	**70**	34	27	43	54	21	51	**101**	**133**	62
Total	115	87	118	105	126	128	123	144	101	104	104	88	111

Percentages which are above the mean for each year are printed in heavy type.

TABLE G. *Cases per thousand of total cases examined in which Klebs-Loeffler was found with Hofmann.*

Jan.	Feb.	March	April	May	June	July	August	Sept.	Oct.	Nov.	Dec.	Total
12	4	7	9	**14**	8	**13**	**14**	7	**12**	4	8	9

1899	1900	1901	1902	1903	1904	Total
21	**17**	5	4	2	4	9

TABLE H. *Cases per thousand of cases examined with Klebs-Loeffler in which Hofmann was found.*

Jan.	Feb.	March	April	May	June	July	August	Sept.	Oct.	Nov.	Dec.	Total
47	15	26	**35**	**48**	31	**45**	**49**	25	**36**	15	30	33

1899	1900	1901	1902	1903	1904	Total
62	**55**	17	16	10	18	33

ANKYLOSTOMIASIS.

By A. E. BOYCOTT, M.A., M.D., B.Sc.

(*Read: Friday, March 17th,* 1905.)

In trying to combat the ravages which are produced by any of the various infective parasitic disorders to which man is exposed, it is of the first importance that the sanitarian should be in possession of full information as to the life-history of the parasite which is the causative agent, and especially that part which is spent outside the human body. Without such knowledge, it is difficult to plan, still more difficult to put into effect, any devices for the undoing of these parasites other than those of an altogether exploratory or empirical nature. Some parasites, such as the *gonococcus*, have, we believe, no saprophytic existence, and no other host than man; remedial measures are perhaps neglected, but they are none the less simple and obvious. In other instances, such as yellow fever, our knowledge of the physiology of the life of the causative agent has been gained in advance of our acquaintance with its morphological nature; it is, however, sufficient to give us good expectations that material progress in the suppression of the disease may continue to be made. In yet other cases—and this class comprises the majority of infective disorders—we know practically nothing of the life of the parasite outside the body of man, and perhaps of certain animals, and any efforts which may be directed against the *B. typhosus* or the *pneumococcus* are necessarily, to a large extent, of the nature of vague experimentation.

The greater part of mankind live in the tropical and sub-tropical parts of the globe. Taking the world as a whole, the two fundamental diseases to which they are here subject are malaria and ankylostomiasis. Until the history of the malarial parasite in its life outside the human body was known, the only weapon in our possession was at once empirical and purely defensive; those great researches of Ross have enabled us to plan a philosophical attack in a practical and, we hope, an efficient manner.

Our knowledge of the morphology and physiology of the

life-history of *Ankylostoma* is still far from complete in many details. In the main outlines, however, it is sufficient to enable us to trace a close connection between the habits and mode of life of the parasite on the one hand, and the whole range of its pathology on the other, and it is from this point of view that I should like to consider the subject of my paper. In the first place, I propose to briefly consider the life-history of *Ankylostoma*; then to review the conditions which may influence this history: and, finally, to consider the applications of these facts to sanitation.

The full-grown sexual *Ankylostoma* inhabits the upper part of the small intestine in man. There are two known species—one, known as *A. duodenale*, is found in Europe, Egypt, etc., while the other, *A. americanum*, has recently been differentiated from the old-world species by Stiles in the United States. The details of the life-history of this latter species have not been fully worked out, but, as far as our information goes, it does not differ from that of the older species, and the effects produced seem to be identical. In case, however, further research reveals any important differences, it is as well to say that the following account refers to *A. duodenale*. The worms are found in the intestine in numbers which vary from two or three to many hundreds. The females lay eggs in very large numbers, and in consequence the stools of an infected individual are often crowded with eggs.

As seen in fresh fæces, the eggs are characterised by a thin, clear, colourless shell, about 60 μ by 40 μ, of a regularly oval outline. The greyish contents are easily seen, and the egg when first passed consists as a rule of four segments. Under suitable conditions, segmentation proceeds very rapidly, and a morula stage is soon reached. The egg may contain an active vermiform embryo in 18 hours, which shortly escapes from the shell. The newly-hatched larva is about 0.27 millimetres long. In favourable surroundings growth proceeds rapidly, and in another 24 hours the larva has moulted, and attained a length of 0.55 millimetres. After a second moult, the beginnings of a definite "chitinous" sheath can be readily made out; and a third moult brings the larva to a final measurement of about 0.6 millimetre. The sheath is now very well developed, and to a certain extent, especially at the head and tail, the larva is free to move within it. This stage is known as the "encapsuled" or "full-grown" larva, and is one of the fundamental landmarks in the cycle of development, representing as it does the adult saprophytic phase of

the parasite. So long as it does not gain entrance to the human body, no further growth takes place; the larva remains asexual, and no multiplication of individuals occurs. If, on the other hand, the larva can obtain a lodgment in the human intestine, development soon proceeds; rudimentary sexual organs appear, and after a series of moultings the adult parasitic stage is reached, with sexual differentiation into male and female individuals, and a rapid multiplication of individuals.

The important points in this life-history are, firstly, that there is no intermediate host, and, as is usual with these higher parasites, the infection of each host is specific for each parasite: thus a dog *Ankylostoma* cannot infect man, nor a human *Ankylostoma* a sheep. In the second place, there is no multiplication of individuals, either within or outside the host. Such increase in numbers can only be brought about by a due and alternating succession of parasitic and saprophytic phases.

The statement, originating, I believe, with Giles and since repeated by several observers, that *Ankylostoma* has a sexual stage outside the body, are to be received with great caution. Their results are readily explained by the assumption—perhaps unjustifiable—that they were not dealing with pure cultures. There are a number of small, entirely saprophytic, nematode worms which are of very general occurrence in earth. The eggs and larvæ of some of these closely resemble those of *Ankylostoma*; the adult sexual forms are, however, quite different, and appear to be exactly similar to the worms figured by Giles and others. These earth nematodes flourish and multiply to an extraordinary extent in fæces to which they may gain access; and I have found them in such situations, as well as in uncontaminated earth, in many English mines, both coal and metalliferous, infected and non-infected. Their frequent presence, and the close resemblance which the larvæ of some of them bear to undoubted *Ankylostoma* larvæ, vitiate also the value of observations on the occurrence and habits of (so-called) *Ankylostoma* in its natural saprophytic habitat. On the experimental side, numerous experiments have, in my hands, given uniformly negative results, and in no case has any sexual form been observed in cultures made from fæces which had not been exposed to contamination from the earth. Similar cultures made from material containing *Ankylostoma* eggs, which had been picked off the ground, showed on many occasions sexual forms of small nematodes belonging to the family *Anguillulidæ*, but only

the larvæ of *Ankylostoma*. Many different methods of culture were tried—aerobic and anaerobic—with duodenal mucosa and various extracts made from it, with blood, at different temperatures, in acid and alkaline media, etc ; but genuine *Ankylostoma* larvæ could never be persuaded to make any further advance in development from the encapsuled stage.

The chances of multiplication are further diminished by the fact that it is only at a certain point of the saprophytic development that *Ankylostoma* becomes capable of infecting its host. It was clearly shown by Leichtenstern that the eggs and young larvæ are not infective ; he found that eggs appeared in the stools only after the full-grown encapsuled larvæ had been administered to the subjects of experiment. I would direct especial attention to this point, which is of unique importance in the consideration of the distribution and epidemiology of the disease.

The time-relations of these changes are also of practical importance. From the fresh egg to the encapsuled larva takes at the least four or five days, and usually longer. The second period of growth, within the host, from the encapsuled larva to the sexual adult, occupies about five weeks. The possible length of life at each stage is less well defined : if the eggs are exposed to conditions which are unsuitable for their immediate development they do not live very long—four or five weeks, perhaps, often only a few days. The encapsuled larva is capable of a very prolonged saprophytic existence. I have been able to keep such larvæ alive in the laboratory at room temperature for more than twelve months. During the whole of this period they lived in an ordinary Petri dish, with no more nourishment than that contained in the fragment of fæces from which they were originally hatched, and in the London tap-water which was added from time to time to make good the loss by evaporation. In their natural habitat they are probably capable of living for a still longer period. The adult parasitic form is also long-lived. Stiles found several cases of infected children in a home in the United States, which was situated outside the endemic area. They had been brought from an infected district more than six years previously, and it is not possible to believe that they had been re-infected during their residence in the well-conducted institution in which they lived. Another interesting example of the duration of the infection came to the notice of Haldane and myself in Cornwall. A youth worked in the infected area—which is here strictly confined to the

underground workings of the mines—in 1898; he soon became anæmic, and gave up the employment. When we saw him at the end of 1902, he had spent the intervening four years in driving a butcher's cart, and had not been even to the surface workings of a mine; yet we were able to find a few eggs in his stools.

A knowledge of the paths of infection gives us little assistance in our endeavours to suppress the parasite. It has been proved long since by direct experiment that the mouth affords for *Ankylostoma*, as for other parasites, an easy mode of access to the intestines; and, until recent years, this was believed to be the only path of infection. Looss, and after him several other observers, have shown, however, that the encapsuled larvæ are capable of penetrating the unbroken skin *viâ* the hair-follicles. It is stated that they are then carried by the blood stream to the lungs, where they form emboli. Gaining access in this way to the air passages, they pass up the trachea and down the œsophagus, and so find their home in the small intestine. If any part of the surface of the body may thus afford a possible point of infection, it is clear that no direct indication is afforded in the matter of prevention; and the brilliant researches which have led to this notable addition to our knowledge are scarcely other than depressing to the sanitarian.

There is, I think, no reason to doubt that infection may, and does, occur by direct reception of the larvæ into the stomach, and thence into the small intestine. Experimentally, encapsuled larvæ may survive for two or three days in strong mixtures of pepsin with hydrochloric acid up to 0.5 per cent., and in alkaline trypsin solutions. If swallowed larvæ pass through the walls of the pharynx or œsophagus, in order to undergo development before facing the ordeal of gastric juice (which is by no means always present in the stomach), it is difficult to see why the interval between infection and the appearance of eggs in the stool should be so much longer in a skin (70 days) than in an alimentary infection (30 days). My own experience is that, 18 months after having made entirely ineffectual attempts to produce an infection through the skin, I became infected at once through the mouth after an accident with a pipette.

We have next to consider the influence of various external conditions on this cycle of development.

1. *Temperature.*—The eggs are killed in about twenty-four hours at a temperature of 40 deg. Cent. (104 deg.

Fahr.), or at a nearly freezing temperature. They may hatch at any temperature from 16 deg. Cent (61 deg. Fahr.) to 38 deg. Cent. (100 deg. Fahr.); in an ordinary hot incubator (36 deg. or 37 deg. Cent.), the eggs often die without hatching, but a few larvæ generally hatch out in about twenty-four hours, and then rapidly die away. The optimum temperature would appear to be about 24 deg. Cent. (75 deg. Fahr.); at rather higher temperatures the eggs hatch more quickly, but the mortality among the young larvæ is high, and many fail to reach the encapsuled stage. The determination of the lowest temperature at which complete development may take place is a point of the greatest practical importance. Below 13 deg. Cent. (15 deg. Fahr.) the eggs will certainly not hatch; but I have twice succeeded in hatching the eggs and growing the larvæ to the encapsuled stage, at a temperature which was generally 15.9 deg. Cent. (60.6 deg. Fahr.), and never exceeded 17 deg. Cent. (62.5 deg.) I may mention that these experiments were made in a Hearson incubator, and that the temperature was noted carefully at very frequent intervals during the whole observation. Several lots of eggs failed to hatch at this temperature, or if hatched failed to develop to the encapsuled larvæ, and there is no doubt that such a low temperature is by no means favourable to *Ankylostoma*. A free and full development is, I think, seldom shown at temperatures below 20 deg. Cent. (68 deg. Fahr.).

Temperature has also a very marked influence on the rate of development. At 35 deg. Cent. free larvæ may appear within twenty-four hours, and encapsuled larvæ in four days; while at 16 deg. Cent. the eggs may take three to seven days to hatch, and encapsuled larvæ may not be present for a fortnight.

Once the larvæ have reached the encapsuled stage, they are relatively indifferent to temperature. I have never succeeded in keeping them alive for more than a few days at 37 deg. Cent., while at room temperature (10 deg. to 20 deg. Cent.) they live for many months. They appear to live equally long at 25 deg. Cent.

2. *Moisture.*—A certain amount of moisture is necessary at every stage, and "ordinary" drying at room temperature kills eggs and larvæ in an hour or two. On the other hand, excessive wetness is fatal to the eggs. Infected fæces covered with a layer of water $\frac{1}{2}$ in. in depth will never hatch at any temperature, and die in about a

week.* The larvæ, on the contrary, prefer to live in water, and the most succesful method of hatching which I have tried is one in which the fæces are kept damp, but not submerged, while they are surrounded with a moat of water into which the newly-hatched larvæ may escape.

3. *Oxygen*—These differences in the relation of *Ankylostoma* to water at different stages of development are explicable, I think entirely, by variations in oxygen requirement. The eggs will only hatch in the presence of a very free supply of oxygen. A gram of infected fæces corked up in a 20 cc. test tube fails to show any larvæ after exposure to a suitable temperature. The inhibitory action of water appears to depend on the relative exclusion of oxygen, for if air be bubbled through a mixture of infected fæces and water at 25 deg. Cent. larvæ hatch out (though only in small numbers) in three or four days. If hydrogen is bubbled through a similar mixture, no development takes place, so that the result obtained with air is not due to the mechanical disturbance. The encapsuled larvæ, on the other hand, show anaerobic capacities of an order which is somewhat remarkable in an animal so high in the zoological series. They will live and exhibit the most lively movement for three weeks in an atmosphere of hydrogen at 17 deg. Cent., and for at least eight days in carbon dioxide, or in a vaccum.

Oxygen is, then, necessary during active growth, but not during the resting stages. Experiments in the anaerobic development of the larvæ showed that development did not proceed unless oxygen was present, though the young larvæ were not immediately killed. These facts are in complete accord with the observed inability of the eggs or young larvæ to produce an infection if taken into the alimentary canal.

4. *Chemical Agents.*—The most exaggerated views have been held on the supposed extraordinary powers of resistance to antiseptics exhibited by *Ankylostoma* larvæ. It is entirely a question of the time factor. It is quite true that the encapsuled larvæ may exhibit active movements for a quarter of an hour after immersion in a 4 per cent. solution of corrosive sublimate, but they were surely killed in four hours in a 0.1 per cent. solution of sublimate, 0.25 per cent.

* After repeating this experiment a number of times with the uniform result mentioned above, I have lately observed an exception in which a few dead one-day larvæ were found in a preparation which had been continuously covered with two inches of water at room temperature.

izal, or 1 per cent. carbolic acid. In twenty-four hours, 0.1. per cent. izal or 4 per cent. sodium chloride will kill the adult larvæ. The explanation of the marked resistance shown by these larvæ when first exposed to the action of the poison is due to the presence of the thick sheath, and it is only after a certain time that this protection is penetrated. Young larvæ, with a much thinner coat, are more easily killed, and in this way the application of a 2 per cent. solution of sodium chloride to infected fæces will prevent the development of encapsuled larvæ, though 6 per cent. is required to prevent the eggs hatching, and 4 per cent. to kill the encapsuled stage.

Bruns found that the more he diluted fæces with water, the quicker and more complete was the development of larvæ. All observers have noticed that occasionally no larvæ can be obtained from fæces full of eggs. This seems to be due to the presence of some substances in certain stools which kill either the eggs or young larvæ. I have found that aqueous extracts of some fæces will kill the newly-hatched larvæ. Why this is I do not know. It is not a question of the reaction. Bruns' view that it is due to the plasmolysing effect of salts can hardly be correct, since fæces contain hardly any soluble salts.

5. *Light.*—Sunlight is said to be quickly fatal to the larvæ. My own experiments on this point gave entirely negative results, but the only sunlight available was that which may be had in London.

It will now be necessary to consider shortly the relation of the infected individual to the infecting agent.

The typical result of *Ankylostoma* infection is an anæmia of greater or less severity. Definite data on the length of time after infection which must elapse before well-marked symptoms are produced are not available, but there are fairly clear cases in which a very profound degree of anæmia has been reached within three months of infection. In the intervening period symptoms are indefinite, but certain "dyspeptic" pains without any association with the taking of food are often present, and have, I think, a definite connection with the presence of the parasite in the small bowel. In many cases a skin affection (botches, bunches, gourmes, mazamorra, coolie-itch, etc.) of varying severity will be present. The degree of anæmia varies from a diminution of 10 or 20 per cent. in the percentage oxygen capacity of the blood, to a condition of the blood which is not exceeded in severity even in the worst cases of perni-

cious anæmia. Patients in the latter condition are, of course, helpless, and frequently die unless treated. Even the former class may exhibit some symptoms of dyspnœa on severe exertion, and their working capacity is thereby distinctly—though perhaps only slightly—diminished. Many cases seem to reach a condition of moderately severe anæmia, beyond which they do not sink. With a percentage oxygen capacity diminished by one-half, they may drag on for years in a state of constant ill-health and inability to do their share of work. The actual destruction of life caused by *Ankylostoma* is considerable enough, but the chronic illness which ensues in neglected and unrecognised cases is responsible for far more unhappiness.*

One important point in the pathology of infective diseases is illustrated particularly well by ankylostomiasis. This is, that many of those who harbour the worm suffer from no symptoms of disease at all. The percentage of sick people in a series who are about equally exposed to an infection varies a great deal with different infections, as well as with different series of possible subjects. From the demonstrated infection of 2, or 5, or 10 per cent. of a population exposed to water- or milk-borne enteric fever, there is every gradation under varying circumstances to the destruction of half a population by plague, sleeping-sickness, or kala-azar. Now, in these instances, we are to a very large extent compelled to judge of the infection of the individual by the obvious presence or absence of disease. In *Ankylostoma,* however, we have a parasite of such dimensions that the criterion of its presence may, if necessary, be made entirely macroscopic, and we can ascertain very clearly what proportions of a population are on the one hand infected, and on the other hand suffering from definite disease. It has been found that in localities which are especially favourable for the worm, practically every individual who is exposed to infection actually harbours the parasite. Dobson found eggs in the fæces of 75 per cent. of the natives in Assam; and in one of the Cornish mines, after examination of about a quarter of the underground hands, taken at random, I failed to obtain satisfactory evidence that a single man was without the worm. Yet, at the worst, not more than 10 per cent. of the men were actually ill, and the great majority expressed themselves

* The truly appalling state of a population saturated with neglected *Ankylostomiasis* is shown very well in the *Report of the Commission for the Study and Treatment of "Anæmia" in Porto Rico*, by Ashford, King, and Ingaravidez. San Juan, P. R. 1904.

to be in perfect health. Of 21,612 men who were found to have eggs in their stools and received specific treatment in Westphalia, only 6.8 per cent. showed definite anæmia. These considerations, I think, support the suggestion that the apparent partial incidence of many infections depends more upon the resistance of the infected individual than upon the mere inclusion of the parasite within his body.

One has, then, to distinguish two classes of infected persons—those who have *Ankylostoma* infection, and those who have the disease ankylostomiasis. Without entering upon a purely pathological discussion, it is perhaps worth mentioning that the tissues of the former class—who may be called immune—are not absolutely indifferent to the presence of the worm, since the blood in these cases shows, as I know from personal experience, the somewhat characteristic changes in the leucocytes to a degree which on the whole is greater than that found in those who are actually anæmic.

The variation seen in the degree of illness produced is, then, due in part to variation in the natural resistance of the individual to the toxic products of the worm. It is also due to variations in the magnitude of the infection. Cases are well established in which a fatal issue has ensued upon the presence of comparatively few worms in the bowel. Perfect health, on the other hand, is not inconsistent with the presence of hundreds of parasites. There is, however, some relation between the degree of anæmia and the amount of infection, and especially the frequency of infection. Individuals exposed to infection under conditions which are eminently favourable to the worm on the whole suffer more severely than those who only meet with the extra-corporeal stage where it has to struggle for existence. These considerations are in harmony with the fact that the anæmia is not due to the abstraction of blood by the worms. This has been clearly established by the investigation of the blood-volume of Cornish patients by Haldane, and is supported by many other details of the infection.

Such is a brief account of the life, habits, and effects of *Ankylostoma*. From these data it is possible to deduce a satisfactory, if not an entirely complete, explanation of most of the facts which are the result of epidemiological observation rather than that of laboratory experiment. Since both lines of investigation lead us to the same ends, it is unnecessary—as it would perhaps be

undesirable—to consider which of the two gives us the better indication for practical hygiene.

Ankylostoma may infect either men or places. The infection of men is immediately the concern rather of the clinician and the pathologist than of the sanitarian. There are, however, two points which require emphasis. I have already laid stress on the fact that many individuals harbour the worm without showing any signs of obvious illness. Yet such persons are potential carriers of infection to other places, no less than those who are actually ill. The healthy "worm-carrier," indeed, is the greater factor in the dissemination of the worm by his more facile locomotion. Such people, too, may remain infective for years after they have removed from an infected locality. The parasite may thus travel all over the world in the persons of those upon whom no suspicion would be likely to fall, and who would, in most instances, be themselves entirely in ignorance of their dangerous character. On the other hand, an infected individual cannot spread the infection before his own parasites have commenced to lay eggs. This favourable point is more than counterbalanced by the fact that, since the diagnosis in the ordinary way rests on the discovery of these eggs in the stools, he would pass even a specific medical examination during the same period.

For the infection of places, three conditions are obviously necessary. Firstly, that infected fæces should be deposited; secondly, that the place so contaminated should present those combinations of temperature, moisture, and æration, which have been already defined as necessary for the development of the infective stage of the worm from the egg. And thirdly, that an interval of time should elapse between the deposition of the eggs and the development of the infectivity of the place: as, however, this interval may be so short as four or five days, this condition does not come to be of any greatp ractical importance, though it precludes a constantly-recurring cycle of auto-infection.

It is, of course, possible that the encapsuled larvæ might be carried from place to place as such. Under these circumstances, conditions of temperature, etc., play a much less important part. The inability of the larvæ, however, to withstand drying renders this mode of transmission of no practical importance, except over very short distances.

The first condition, that fæces should be deposited in such positions that human beings shall thereafter come into contact with them, is fulfilled all the world over with varying degrees of completeness. The majority of man-

kind makes no special provision whatever for the disposal of excreta. Among relatively savage races this is perhaps not unreasonable; but it is surprising to learn that among, *e.g.*, the farming class in the southern parts of the United States, even the most elementary privy accommodation is unknown. With permanent houses the result is obvious, and the whole of the soil in the immediate neighbourhood of their dwellings is saturated with fæcal contamination, growing worse and worse as time goes on. And it is just these very parts of the earth with which the inhabitants come into intimate daily contact in the pursuit of agriculture. A nomadic people are in a far better position, as their filth is periodically left behind them, and the custom among the Burmese of having a fresh capital for each king finds ample justification in considerations of public health alone. Greater care is taken in the disposal of excreta in this country, I suppose, than in any other; yet a brief inspection near any large centre of population will soon show anyone whose observation is sharpened by interest that there is abundant opportunity for the careless or unwary to come into contact with human excrement. That the population of London have not infrequent contact with stale human fæces is shown by the fact that the eggs of the worm *Trichocephalus dispar* were found in the stools of rather more than 10 per cent. of 250 patients taken at random in the general wards of Guy's Hospital. Dr. French is still continuing this inquiry, and I am indebted to him for permission to mention the main result now. It is one of great interest, for in *Trichocephalus* we have a parasite which is not capable of any multiplication outside the body, and one, too, which (as far as is known) can only become established in the bowel by ingestion with the food of eggs which have lain outside the body for a time sufficient to enable the larva to develop. The prevalence of this harmless parasite gives, I imagine, an excellent minimal value for the excremental contamination of the intimate surroundings of a population. I have found, for instance, that three-quarters of the men who work in the abominable filth of some of the Cornish mines, are infected with *Trichocephalus*; and even higher percentages have been constantly found to accompany the elementary habits of the natives of the East.

If, then, fœcal contamination were the main factor in the occurrence of ankylostomiasis, the disease would be prevalent all the world over. The second condition is, however, the one which determines the geographical and topical

incidence. Epidemiological observation confirms the suspicion which is aroused by laboratory experimentation—that *Ankylostoma* would be found to be prevalent only where the eggs, on leaving the host, find those conditions of temperature, moisture, etc., which enable the larva to develop to the infective stage. Generally speaking, the geographical distribution of *Ankylostoma* is a question of temperature. It has been found in almost all parts of the tropical and sub-tropical world, and in all probability a short search would soon reveal its presence in those districts from which it has not yet been reported. In Europe it occurs among the general population south of the Alps; it is very common in northern Italy, and occurs in Hungary. It is probably common enough in Spain, though there are no details available. In northern Europe it has been found in those areas in which the necessary constant warmth has been provided by artificial means; the coal mines in Westphalia, in northern France, and in Belgium, and the metalliferous mines in Cornwall, have all suffered more or less severely from the ravages of the worm. The temperature in the underground workings of most extensive mines is high enough, at least in parts, to enable the eggs to hatch and the larvæ to grow to the encapsuled stage. Taking the possible lower limit as 16 deg. Cent., and the optimum as 25 deg. Cent., as defined by the experiments mentioned above, we find a striking confirmation in the following Table, showing the numbers per thousand of men suffering from illness in the different mines of the Westphalian coal-field, the mines being grouped according to their temperatures :—

Temperature.	Number of Collieries.	Number of Men Employed Underground.	Cases of Anæmia per 1,000 Men Employed Underground.
Less than 20 deg. Cent. (68 deg. Fahr.)	151	104,637	0.5
20 deg. to 22 deg. Cent. (68 deg. to 72 deg. Fahr.)	45	43,710	2.5
22 deg. to 25 deg. Cent. (72 deg. to 77 deg. Fahr.)	33	39,836	11.7
Over 25 deg. Cent. (77 deg. Fahr.)	12	9,853	39.9

These figures show that illness does not occur to any extent unless the temperature is such that the conditions are distinctly favourable for the development of the worm. In the inquiry into the prevalence of *Ankylostoma* in the Belgian coal mines, attention was paid rather to the number of infected men than to the number of cases of illness; and it was found that, under conditions of temperature at which development is possible, though not encouraged, a considerable number of miners became infected. Thus in sixteen mines, with a temperature of 15 deg. to 20 deg. Cent., the percentage of infected men varied from 4 to 52, though the cases of actual illness were relatively few.

It is perhaps interesting to consider the possibility of *Ankylostoma* living on the surface in this country. The Greenwich Records for Fifty Years show that the mean daily temperature exceeds 60 deg. Fahr. on an average of 71 days in the year; the average number of days per annum on which the temperature rises above 70 deg. Fahr. is 77, and on nearly four days per annum does it fail to fall below 60 deg. Fahr. The average number of days on which the temperature falls to freezing point is 56, and on nearly five of these it remains continuously below zero for 24 hours. Though the Greenwich Records represent the temperature of one of the colder localities in England, it would follow that *Ankylostoma* could only live under the most adverse circumstances, and each winter season would probably put a stop to the infection of any surface locality. Experiment shows, however, that it is quite possible for infected fæces left in the open in London to give rise to encapsuled larvæ. On two occasions in the early part of July of last year (1904), I found fully-developed larvæ in infected stools which had been left in the open air, and out of the direct influence of the sun, in a yard at Guy's Hospital. Similar experiments made during the period November to June gave uniformly negative results.

The question of moisture plays a less prominent part in the geographical prevalence of the worm, since few localities fail to provide the small amount which is absolutely necessary. The melancholy history of the Westphalian pits illustrates, however, the importance of moisture in the degree of vigour which the infection may display. The following Table gives the number of cases of marked anæmia observed in the Westphalian colliers from 1896 to 1902:—

1896	...	107	1899	...	94	1901	... 1030
1897	...	113	1900	...	275	1902	... 1355
1898	—	99					

In 1900 a system of watering the roads was made compulsory, in order to prevent explosions of coal-dust. This desirable object has been, I believe, obtained, but only at the price of an epidemic of ankylostomiasis, which has cost the coal industry many thousands of pounds, and which is still a source of no inconsiderable trouble and danger.

The only practical application of the need of oxygen shown by the developing embryo which is of material importance is this—that fæces deposited in and covered by water are safely disposed of, since no development will then take place. The anærobic qualities of the adult larvæ and its power of flourishing in water, render its transport by moving water possible and easy; in this way, infection may be brought over a short distance from an area of high temperature to one where the eggs would not hatch. Such circumstances as would render such a possibility of practical importance are of frequent occurrence in mines. When in water and undisturbed, the larvæ seem to quickly sink to the bottom; and since the finer sediment with which they are mixed in the polluted mud or dirt where they develop settles more slowly than the larvæ themselves, water which is perfectly clear on naked eye examination is probably free from infection.

The relation of the larvæ to poisonous solutions may occasionally play a most important part in the occurrence of the disease. An excellent example of this is found in the history of the Levant mine, in Cornwall. The present workings of this mine are to a large extent under the sea; the ventilation is very imperfect, and the temperature very high, rising from 71 deg. Fahr. at the bottom of the downcast shaft to 90 deg. to 93 deg. Fahr. in large areas of the deeper workings. The levels, ladders, etc., are wet and muddy, and the fœcal contamination extensive. A considerable number of men have returned to work in this mine from infected districts abroad, and some men who were actually known to harbour *Ankylostoma* had worked here for a time. The conditions, in short, for the introduction and spread of the disease were at least as favourable as those prevailing in other Cornish mines which were severely infected. Yet no trace of *Ankylostoma* could be found. The reason for this evidently lay in the nature of the water which permeates the mine. Some of this flows in from the land side, and is ordinary spring water, but

most of it is derived from the sea by leakage through the roof into the upper levels. As a result, the average content in salt of seven samples of water from different parts of the mine was 2.23 per cent., an amount which, as has been shown above, is sufficient to destroy the newly-hatched larvæ. There is no doubt that this mine owes its freedom from *Ankylostoma* to the somewhat undesirable thinness of the roof, and the resulting salinity of the water.

It will now be apparent that our knowledge of the life-history of *Ankylostoma* is sufficiently accurate and sufficiently—though far from altogether—complete to afford us very clear and definite indications in the matters of prevention and treatment.

Prevention of ankylostomiasis is secured by any system of disposal of excreta which is in accord with Western notions of common cleanliness.

The treatment of ankylostomiasis may be either the treatment of the infected individual or of the infected place. The former is beyond the province of the present paper; but, as it in practice becomes an integral part of the treatment of places, brief mention of it is necessary. We have, fortunately, in thymol a drug which acts as an exceedingly efficient anthelmintic for *Ankylostoma*, if used in the proper way. The first necessity is that adequately large doses should be given: there is a rather prevalent feeling that thymol is an extremely dangerous drug, and a consequent tendency to "be on the safe side" in the doses which are given. Small doses are "on the safe side" for both the host and the parasite, and in consequence no good results. The second necessity is that treatment should be repeated as often as is necessary to kill all the worms; this can only be judged of by very careful microscopical examination of the stool for eggs after each dose. It is worse than useless to treat an infected individual without taking the trouble to gain an accurate knowledge of the result: one dose often fails to kill all the worms, though the patient's general health may be much improved. As a consequence further treatment is not insisted on, and the worm-carrier, efficiently isolated while seriously ill, is enabled to go abroad and spread the disease still further.

The treatment of infected places opens up a very large, and in many places still untried, field. The indications are clear enough, but in practice the feasibility of following them out is of necessity an important consideration. It is obviously necessary to prevent further contamination, on the one hand by arranging that all fæces shall be

disposed of in an adequate manner; on the other, by treating everyone who harbours the worm until eggs are no longer present in the stools. Re-infection of the locality is thus prevented. How to destroy the infection already present must depend on local circumstances. It is practically certain that the larvæ, long-lived as they are, must die out sooner or later, and, since no reproduction of individuals takes place outside the body, evacuation of the locality would in the end be efficacious. If this is impossible, treatment with poisonous solutions might, I am convinced, be undertaken in some instances (notably, of course, in mines) with some prospect of success. In the case of coal-mines, evacuation would result in the production of an anaerobic atmosphere in which, after some time, the larvæ would perish.

Where the infected area is circumscribed, as in mines or tunnels, prevention is easy. The success with which the worm has been excluded from the Simplon Tunnel workings is a great testimony to the vigilance of those responsible for the medical care of the workmen; it is also conclusive proof that such disasters as that at the St. Gothard twenty years earlier are quite preventible. The cure, however, of even a limited underground area is a matter of difficulty, as the Westphalian experience has shown. A system of frequent examination of all underground workers, followed by treatment of those found to be infected, has been combined with apparently efficient efforts to overcome the peculiar prejudice in the matter of defæcation which seems to be common to all miners. By this means the amount of actual illness has been reduced to *nil*. It is, however, disquieting to learn that all the efforts which have been made during the last four years have failed to altogether eradicate the infection. At one colliery, for instance, the percentage of infected men was 20 per cent. at the first round of examination and treatment, 13 per cent. at the second, 8 per cent. at the third, and 3.6 per cent. at the fourth. The figure remained, however, about the same at the fifth, sixth, and seventh examinations. This persistence is probably accounted for by the fact that the pit is still infected, though fresh contamination has been stopped. There are, also, a certain number of errors of diagnosis, since worms may be present in the intestine, and yet no ova may be found in the stools; and a few cases have been met with in which the most vigorous anthelmintic treatment has failed.

Artificial alterations in the temperature and moisture

are in all cases extremely difficult, and in most cases quite impracticable. They will also do no more than influence the development of the eggs. The same end is attained more simply by securing that there should be no eggs. In a few cases dryness may be aimed at as an auxiliary measure, and in still fewer a slight diminution of temperature may be obtained, which may help to turn the scale against the worm.

In the tropics, where the infected locality is co-extensive with the inhabited area, little can be done beyond treatment of cases of definite illness as they arise, and prevention of a certain amount of infection by the protection of bare feet and legs. It seems to be beyond hope that the natives should be induced to assist by disposing of their excreta in appointed and suitable places.

It would seem unnecessary to remark that the recognition of *Ankylostoma* as one of the most potent causes of illness and inefficiency in hot climates is the first step in the way of treatment. It has, however, always been characteristic of the disease that its true nature should long remain unsuspected. In Puerto Rico, for instance, about one-quarter of all deaths were certified as due to "anæmia," yet it remained for Ashford to point out, in 1900, that the real cause was *Ankylostoma*. In the same way, it was not till 1902 that Stiles showed that ankylostomiasis is extremely prevalent in the southern parts of the United States. Yet, again, the worm caused much illness among the Cornish miners from 1894 onwards; but the real cause of "Dolcoath anæmia" remained unsuspected, and necessarily untreated, until it was pointed out at the end of 1902 by Haldane.

NOTE.—Further information with full bibliographies may be found in—*Report upon the Prevalence and Geographic Distribution of Hookworm Disease in the United States*, by C. W. Stiles, Bull. 10, Hyg. Lab., U.S. Public Health and Mar.-Hosp. Service, Washington, 1903, and *L'Ankylostomiase*, by A. Calmette and M. Breton, Paris, 1905.

[From THE JOURNAL OF HYGIENE, Vol. V. No. 3, July, 1905.]

A CASE OF SKIN INFECTION WITH ANKYLOSTOMA.

By A. E. BOYCOTT, M.A., M.D.

(*From the Lister Institute of Preventive Medicine.*)

In view of a previous failure[1] to produce a skin infection with *Ankylostoma*, it seems worth while to put on record the present successful case. Dr J. B. Leathes very kindly offered himself as the subject of experiment. It is important to note that he has never, either before or during the course of the experiment, had anything to do with any *Ankylostoma* material except that deliberately applied to his person for the purpose of this experiment, nor has he been to any infected place or even worked in the part of the Institute where the cultures of *Ankylostoma* larvae have been kept. On March 21st a small quantity of water containing encapsuled larvae of *A. duodenale*, hatched artificially from infected faeces from Cornwall, and 13 days old, was applied to the forearm. After five minutes the arm was bandaged up and left for two hours, at the end of which time the wrappings were removed and the arm thoroughly cleansed. The purpose of the experiment and the precautions necessary were fully appreciated by the subject, and there is no doubt that there was not the slightest possibility of a mouth infection.

A definite eosinophilia was found on April 17 (27 days). Eggs were looked for on May 4 and May 6 without success, but on May 10 (50 days) a few eggs were found. Thymol (40 grains) was given on May 13 (53 days), but on May 22 one or two eggs were still present. On May 27 (67 days), 90 grains of thymol were taken in three 30-gram doses, and on June 6 a single egg was found with some difficulty. This failure to effect complete cure immediately after infection is possibly due to the varying times taken by different larvae to reach the intestine.

[1] This *Journal*, vol. iv. 1904, p. 89.

The symptoms observed were slight, though definite. The morning after the application of the larvae half-a-dozen small roundish patches of erythema were present in the treated area; these were not raised and faded completely on pressure. They made no further progress and in a few days had completely disappeared. Itching was felt for the first time a few hours after the larvae had been washed off the arm and continued for about a fortnight. Symptoms of "dyspepsia," hardly amounting to more than epigastric discomfort, were noticed after about fourteen days, and lasted till thymol was taken; after this they diminished, but were still present to some extent. The subject of the experiment is normally altogether a stranger to such symptoms, which seem to be just the same as those previously experienced by the writer after infection[1], to which they are doubtless due. Somewhat indefinite symptoms of bronchial catarrh were present from the second week onwards. No anaemia was produced.

The blood, and more especially the differential leucocyte count, was examined from time to time. The chief results are given in the following table:

Days after infection	Haemoglobin per cent.	Leucocytes per cub. mm.	Lymphocytes	Intermediate	Large hyaline	Neutrophile	Eosinophile	Mast-cells
0			20·0	12·4	4·6	61·0	**2·0**	0[2]
14			9·8	14·0	5·2	62·2	**7·4**	1·4
21			29·0	5·0	3·4	58·2	**3·0**	1·4
27	102	8000	8·4	17·6	6·0	53·2	**14·4**	0·4
43			13·2	7·8	5·4	60·0	**13·4**	0·2
44	105	7000	19·6	8·0	3·6	48·0	**20·8**	0
48			12·8	9·6	2·4	38·8	**35·6**	0·8
50		Eggs found						
51			15·6	3·4	0·6	52·0	**28·0**	0·4
52	98	10600	10·8	5·2	3·2	35·2	**44·8**	0·8
53		Thymol grains 40						
55	102	8100	19·6	5·4	2·0	40·0	**31·0**	2·0
64	96	8800	19·2	2·8	2·0	54·4	**21·6**	0
67		Thymol grains 90						
69	100	7800	24·4	5·6	2·0	46·0	**20·8**	1·2
72		7100	13·6	11·6	1·6	56·0	**17·2**	0
95			21·6	4·0	3·2	47·2	**22·8**	1·2

It will be seen from this that a definite eosinophilia was found 27 days after infection and 23 days before eggs appeared in the stool.

[1] This *Journal*, vol. IV. 1904, p. 463; see also C. A. Smith, *Journ. American Med. Association*, vol. XLIII. 1904, p. 592.

[2] Mast-cells were constantly present, though not always found in the leucocytes enumerated. The counts were all made at about the same time of day, fasting.

The eosinophilia subsequently rose to a very high figure (45 p.c.), subsiding somewhat after treatment. The total leucocytes were once observed to exceed 10,000, but even at the height of the eosinophilia there was no marked leucocytosis. Such increase as there was is fully accounted for by the increase of eosinophiles alone.

The following curves compare the time-relations of the eosinophilia in the present case and in the case of mouth infection which has been already dealt with[1]. The rise is rather earlier in the latter, but taking into consideration the fact that a slight increase of eosinophiles was present at the time of infection, there does not appear to be any necessarily significant difference in the two cases. The worm evidently begins to produce the substance to which the eosinophiles react at the

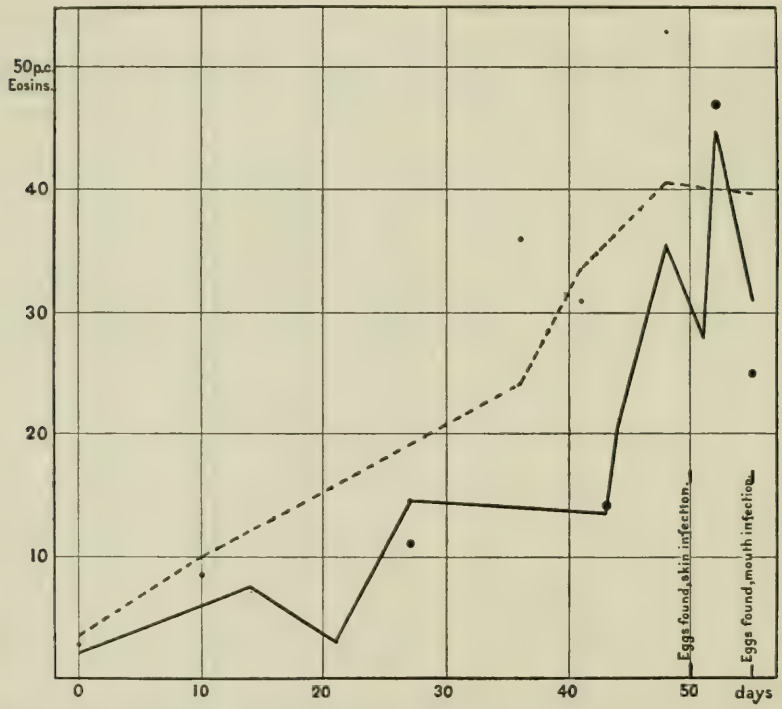

Fig. 1. The ordinates represent percentage of eosinophiles, the abscissae days. The continuous line is the skin, the dotted line the mouth infection. The separate points give the absolute eosinophiles where these were determined.

[1] This *Journal*, vol. IV. 1904, p. 462.

stage in its development which is reached 14 to 21 days after its entry into the body[1].

During the last eighteen months a number of important observations have been made on the skin infection in ankylostomiasis. Looss[2], working with *A. caninum* and dogs, has completed his demonstration by showing that the larvae leave the neighbourhood of the skin in the blood stream, pass through the right heart and are arrested in the lungs, whence they travel up the trachea and down the oesophagus to the intestine. These results have been fully confirmed by Schaudinn[3], who, using *A. duodenale* and monkeys (*Inuus*), found larvae in the blood of the right heart and in the lungs a short time after infection, and adult worms in the intestine from a previous application of larvae to the skin. C. A. Smith[4] has brought about skin infection in man with *A. americanum*; he found eggs in 45 days. This period, as well as that of 50 days found in the present experiment, is much shorter than that previously found by Looss (71 days), and by Pieri (71 days), and is so little different from that observed in mouth infections that any emphasis which has previously been laid on the difference seems to be hardly valid. With *A. caninum* and dogs, Lambinet[5] has shown that eggs appear in the stool in just the same time (20 days) whether the larvae are swallowed, or applied to, or injected under, the skin. Herman[6] has also successfully infected himself by the application of larvae to the skin.

The clinical aspects of the skin eruptions and their essential connection with the disease have been fully described by C. A. Smith[7] in the United States ("ground itch"), and by Ashford, King, and Igaravidez[8] in Porto Rico ("mazamorra"). The natives in the latter

[1] The eosinophilia is extraordinarily persistent. A man who while infected showed 17–19 p.c. still has 11 p.c. though it is more than 30 months since treatment was commenced and fully 20 months since he was restored to health and his stools found to be free from ova on repeated examinations.

[2] Communicated on behalf of Prof. Looss by Prof. Elliot Smith to the meeting of the British Medical Association at Oxford; see abstract in *The Mining Journal*, Aug. 6, 1904, p. 138.

[3] *Deutsche med. Wochenschr.* 1904, p. 1338.

[4] *Journ. Amer. Med. Assoc.* vol. XLIII. 1904, p. 592.

[5] *Bull. de l'Acad. Roy. de Méd. de Belgique*, Jan. 28, 1905. F. Smith, *Journ. Roy. Army Med. Corps*, vol. IV. 1905, p. 335, and H. Liefmann, *Zeitschr. für Hygiene*, vol. L. 1905, p. 349, have also recorded successful skin infections in dogs with *A. caninum*.

[6] *Acad. Roy. de Méd. de Belgique*, procès-verbal, Jan. 28, 1905, p. 8.

[7] *Journ. American Med. Association*, Sept. 19, 1903.

[8] *Report on Anaemia in Porto Rico*, San Juan, 1904. This valuable report shows

place have long recognised the aetiological connection between mazamorra and anaemia, just as in Cornwall the miners knew that dyspnoea followed "New sump bunches." There is an older account of ankylostomiasis which is of especial value and interest in this connection[1]. The author, after a careful review of the epidemiology of the miners' anaemia of the French coal-field, concluded at the time of writing that the anaemia is due to products of the distillation of heated coal. No suggestion had then been made of a parasitic origin. He describes however certain skin affections as an essential part of the disease, which are obviously identical with those seen in Cornwall. They were celebrated in the topical songs of the period and consisted of: "(1) a papular-vesicular-pustular eruption, very painful, called *ampoules*, affecting the hands and feet, and, in a more generalised form, the parts of the body coming in contact with the mine-water and coal-dust. (2) An urticaria, called *gourmes*, forming red indurated swellings up to the size of a bean, extending into the subcutaneous tissue. Each lump lasts 2 or 3 days, and there are successive crops[2]. They affect parts which have been in contact with wet coal and appear 12 to 24 hours after contact. When the urticarial nodules are numerous, they are accompanied by severe bronchial catarrh (*catarrhe des gourmes*)." This last observation is of great interest in view of the recent demonstration that the lungs form a temporary lodgement for the larvae on their way from the skin to the bowel. Skin symptoms do not appear to have been noticed in the Belgian and Westphalian coal-mines.

in a striking manner the fearful havoc which *Ankylostoma* can play in a bare-footed and untreated population. A large proportion of the natives seem to have less than 50 p.c. haemoglobin.

[1] A. Manouvriez, *De l'anémie des mineurs, dite d'Anzin*, Valenciennes, 1878. See also *De l'anémie ankylostomiasique des mineurs*, Valenciennes, 1904.

[2] It has been previously pointed out (This *Journal*, vol. III. 1903, p. 109), that attacks of urticaria, like the general pruritus, may also occur long after the patient is removed from any possibility of fresh infection. See also *Journ. Roy. Army Med. Corps*, vol. IV. 1905, p. 652.

Journal Physiology, Vol. XXXII.
[*From the Proceedings of the Physiological Society*, Dec. 17, 1904.]

On the occurrence of Inactive Arginine.

By E. P. Cathcart.

In a paper which I recently published on the products of "Urotryptic" digestion[1], in discussing the diamino acids I left, owing to a certain degree of uncertainty, the subject of the arginine fraction for future consideration. The main cause of the uncertainty was the difficulty of isolation by means of the basic silver salt, as this compound refused to crystallise out properly. Accordingly, after suitable preparation, the product of this fraction was reprecipitated with phosphotungstic acid, and then on neutralising the basic solution, obtained from the decomposition of the phosphotungstic precipitate, with nitric acid one obtained a fairly abundant yield of crystals, which on further examination proved to be the inactive modification of arginine.

Analysis.

0·2323 grm. subst. gave 0·1344 grm. H_2O and 0·2636 grm. CO_2

	C	H
Found	30·95 %	6·47 %
Calculated for $C_6H_{14}N_4O_2, HNO_3$	30·34 %	6·37 %

There was no sharp melting point but decomposition began at 210°. Kutscher[2] gives 211° as M.P. The melting point of the ordinary active form as given by Gulewitsch[3] is 175°.

The substance yielded no very characteristic salts except the nitrate. I tried to obtain both silver salts corresponding to the two silver salts of the active form, that of arginine nitrate with copper, and the sulphate of the base.

Only once previously has this inactive form been isolated and that was by Kutscher[4] from a digestion of fibrin with pancreas (it may be mentioned that Kutscher has likewise obtained inactive arginine from the active by treatment with sulphuric acid). It is to be noted that in my research I also used fibrin, but whether this has any bearing on the question I am not yet in a position to say.

[1] *Salkowski's Festschrift*, 1904.
[2] *Zeit. f. physiol. Chem.* XXVIII. p. 88.
[3] *Zeit. f. physiol. Chem.* XXVII. p. 189.
[4] *Loc. cit.*

Lister Institute.

Journal Physiology, Vol. XXXII.
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The formation of inactive arginine by enzymes from proteids which yield optically active arginine on hydrolysis with acids. By E. P. CATHCART.

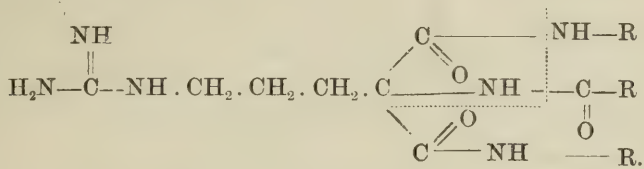
Optically active arginine has been repeatedly recorded as a product of the hydrolysis of proteids by both acids and enzymes. In a recent communication[1] to this Society I showed that inactive arginine resulted from the digestion of fibrin by means of "Urotrypsin," the same proteid on the other hand yielding dextro-rotatory arginine when hydrolysed by sulphuric acid. A similar phenomenon has now been observed in the case of the hydrolysis of coagulated serum by sulphuric acid and by an enzyme found by Hedin[2] in the spleen (lieno α-protease)—the acid decomposition yielding the active base, whereas the enzyme produced the racemic variety.

These results are entirely contrary to what one would expect. The conditions usually associated with racemisation phenomena, namely high temperature and the presence of powerful chemical reagents, are much more nearly realised in the case of acid than in the enzyme hydrolysis, yet it is the latter decomposition which furnishes the racemic arginine. This fact is the more remarkable in view of the general belief that an enzyme molecule is asymmetric.

It is impossible to offer a complete explanation of the observed facts, but at least two possibilities suggest themselves. Firstly, it may be that the optically active arginine complex either before or after liberation from the rest of the proteid molecule is racemised through the agency of the enzyme. Such a change is admittedly difficult to picture but it may be recalled that optically active substances such as atropine[3] and amygdalin[4] undergo partial racemisation at the ordinary temperature under the katalytic action of weak alkali. Secondly, it may be imagined that the mode of splitting by means of acids is not identical with that of enzymes. It is conceivable, for example, that the arginine complex in the proteid molecule does not contain an asymmetric carbon atom,

[1] *Proc. Physiol. Soc.*, Dec. 17, 1904; *Journal of Physiol.* XXXII.
[2] *Journal of Physiol.* XXX. p. 155. 1903.
[3] *Berichte*, XXI. p. 2777. 1888.
[4] *Trans. Chem. Soc.* LXXXV. p. 1512. 1904.

and therefore may under suitable conditions be liberated in the form of the inactive base. A symmetrical grouping which would explain the formation of inactive arginine might be represented as follows, in which R represents other amido groupings:



Under other circumstances however an asymmetric carbon atom may be produced during the course of the hydrolysis, and thus what may be regarded as an asymmetric synthesis can occur.

The experiments are being continued with the hope of elucidating the course of the reaction.

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ON THE PRODUCTS OF DIGESTION OF THE PROTEOLYTIC SPLEEN ENZYME ACTING IN AN ALKALINE MEDIUM.

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Some two years ago Leathes[1] investigated the products formed by the action of the lieno β protease, the enzyme acting in an acid medium discovered by Hedin and Rowland[2] in spleen juice, and more fully investigated later by Hedin[3]. It was accordingly of some interest to see whether the products resulting from digestion with the lieno α protease, the spleen enzyme acting in an alkaline medium, were similar to those discovered by Leathes.

The necessary digestion was carried out with the enzyme, isolated in the manner described by Hedin in his paper, from some 25 ox spleens. The enzyme solution was then added to finely ground and well washed coagulated blood serum suspended in 0·25 % sodium carbonate solution, to which a small quantity of toluol and chloroform had been added. After 7½ months' digestion at 37°, when the increase in non-precipitable nitrogen—as tested for in the tannic acid method—was found to have ceased, the whole digest was decanted into a large basin, faintly acidified with acetic acid, and heated for some time on a water-bath in order to coagulate any proteid left. After removal of the solid matter by filtration the filtrate gave only a slight precipitate with tannic acid. To free the fluid completely from the proteid substance it was further treated with an excess of lead hydroxide and then filtered. On freeing the filtrate from lead by means of H_2S the resulting fluid gave no precipitate with tannic acid, only a very slight biuret reaction, no bromine, but a very well-marked Adamkiewcz-Hopkins reaction.

Separation of the products of digestion.

I. The solution, after concentration, was precipitated with 10 % mercuric sulphate in presence of 5 % H_2SO_4, in order to precipitate any tryptophane present. Hopkins and Cole[4].

II. After removal of the mercury from the filtrate, phospho-tungstic

acid was added for the purpose of separating the monamino and diamino acids.

I. Precipitate with mercuric sulphate. The precipitate formed was allowed to stand for about 12 hours, then filtered off and washed with 5 % sulphuric acid until the filtrate gave no red colour with Millon's reagent. The mercury precipitate was then decomposed with H_2S, the decomposition being carried out several times, at first in cold, latterly in warm acid solution. The filtrate was next treated for the separation of cystin and tryptophane as advised by Hopkins and Cole. The two fractions were worked up separately, but with negative results in both cases. The tryptophane fraction gave the glyoxylic but not the bromine reaction; the substance which was eventually obtained was syrup-like and refused to crystallise under any conditions. The cystin precipitate, which was a very small one, gave the lead reaction, but no crystals could be found.

II. Precipitate with phospho-tungstic acid.

(*a*) *Diamino Fraction.* The filtrate and washings from the mercury precipitate were freed from mercury by means of H_2S, concentrated on the water-bath, and then whilst still hot precipitated with a hot solution of the requisite amount of phospho-tungstic acid in 5 % sulphuric acid. After standing for 24 hours the precipitate formed was filtered off and well washed with a solution of 3 % phospho-tungstic acid in 5 % sulphuric acid and finally dried by suction. The precipitate suspended in water was decomposed with barium hydrate; during the decomposition slight evolution of ammonia was noted, filtered, and the filtrate, from which a small excess of barium was removed by sulphuric acid, then concentrated. The further treatment, for the separation of the hexone bases, was carried out according to the method of Kossel and Kutscher[5].

The histidine fraction, having been precipitated with silver nitrate in the usual way, was well washed with water. The precipitate, suspended in dilute sulphuric acid, was then decomposed with sulphuretted hydrogen, the decomposition being repeated several times. The filtrate from the silver sulphide was next concentrated until its content in sulphuric acid equalled $2\frac{1}{2}$ %, and from this solution the histidine was precipitated according to the method of Kossel and Patten[6] with mercuric sulphate. The free base thus obtained was finally converted into the bichloride.

Analysis. 0·3099 grm. substance gave 0·0574 grm. N.
N found = 18·5 %. N calculated for $C_6H_9N_3O_2 2HCl$ = 18·4 %.

The arginine fraction obtained by the addition of an excess of saturated solution of barium hydrate to the filtrate from the histidine fraction was filtered off and well washed with baryta water and then decomposed as usual with sulphuretted hydrogen in presence of sulphuric acid. The filtrate, from which the sulphuric acid was removed, was concentrated until the solution was of the consistence of thin syrup, then exactly neutralised with nitric acid, and further concentrated and left to crystallise. The crop of crystals obtained was purified by several recrystallisations from water.

Analysis. 0·2477 grm. substance gave 0·1432 grm. H_2O and 0·2816 grm. CO_2.
Found 6·46 % H and 31 % C.
Calculated for $C_6H_{14}N_4O_2HNO_3$, 6·37 % H and 30·34 % C.

On testing the crystals as regards their optical activity they were found to produce no rotation. (The original mother liquor was also tested with the polariscope, it likewise was inactive.) That this was the inactive arginine nitrate was further confirmed by taking the melting point when decomposition was found to begin at 212°. Kutscher[7] gives 211° and I[8] found in a previous sample 210°. The melting point of the active variety as given by Gulewitsch[9] is 175°.

Lysin Fraction. The barium and silver in the filtrate from the arginine were removed by means of sulphuric acid and sulphuretted hydrogen respectively, the filtrate concentrated, and the base reprecipitated with phospho-tungstic acid, the resulting precipitate being as usual decomposed with baryta. The excess of baryta removed by means of CO_2 from the filtrate, which was then concentrated to a syrup and treated with a saturated alcoholic solution of picric acid as long as a precipitate was formed. The picrate, which was boiled out 3 times with absolute alcohol as recommended by Kutscher and Lohmann[10], in order to remove any cholin which might be present (it may be noted here that no cholin was found), was decomposed with 10 % hydrochloric acid, the free picric acid removed by shaking out with ether, the solution then concentrated to small volume, and the lysin bichloride allowed to crystallise out. Crystals subsequently purified by recrystallisation from alcohol.

Analysis. 0·2000 grm. gave 0·2606 grm. AgCl.
Found Cl = 32·22 %.
Calculated for $C_6H_{14}N_2O_2 2HCl$ = 32·42 %.

(*b*) *Monamino Fraction.* The filtrate from the precipitation of the hexone bases with phospho-tungstic acid, freed from the excess of phospho-tungstic acid by means of baryta, was concentrated, during which process some tyrosine along with a little leucine crystallised out. The solution was eventually concentrated to dryness, the crystal mass obtained being then treated according to E. Fischer's ester method. The esters obtained were fractionally distilled in a vacuum of from 14—16 mm. except in the case of the first, which chiefly consisted of ether.

Fraction up to 30°, mostly ether.

Fraction, coming over between 30°—70°, likewise contained some ether. In addition, however, after saponification and concentration of the solution a small quantity of alanine was obtained.

Analysis. 0·1533 grm. substance gave 17·1 c.c. $\frac{N}{10}$ H_2SO_4 = 15·68 % N.
Calculated for $C_3H_7NO_2$ = 15·73 % N.

Fraction 70°—80°. This fraction seemed to consist mainly of amido-valerianic acid, leucine, and some α-pyrrolidin carboxylic acid. In order to obtain the α-pyr. carb. acid this fraction and the following one after saponification were extracted, separately, with cold absolute alcohol (Kossel and Dakin[11]), the alcoholic filtrates united, concentrated and then allowed to dry in a desiccator. The substance obtained was again extracted with cold alcohol and filtrate dried, this extraction was repeated a third time. The final product was converted into the hydantoin derivative, typical crystals being formed.

Analysis. 0·0652 grm. substance gave 0·0329 grm. H_2O and 0·1601 grm. CO_2.
Found = 5·64 % H and 66·97 % C.
Calculated for $C_{12}H_{12}N_2O_2$ = 5·57 % H and 66·67 % C.
M.P. was 143°, same figure given by Fischer.

This same fraction gave, after fractional crystallisation, a fair yield of crystals of amido-valerianic acid.

Analysis. 0·2441 grm. subst. gave NH_3 = 20·3 c.c. $\frac{N}{10}$ H_2SO_4 = 0·02842 grm. N = 11·64 % N.
Calculated for $C_5H_4NO_2$ = 11·96 % N.

Fraction 80°—100°. This fraction contained some amido-valerianic acid mixed with the leucine of which it seemed chiefly to consist, and as mentioned above some α-pyr. carb. acid. The leucine crystals after repeated fractional crystallisations were obtained were quite pure.

Analysis. 0·1258 grm. subst. gave 0·01358 grm. N = 10·79 % N.
Calculated for $C_6H_{13}NO_2$ = 10·69 % N.

Fraction 120°—125°. This fraction was found to contain glutamic acid, phenylalanine and probably a small quantity of aspartic acid. To obtain the phenylalanine this fraction and the following one were treated, before saponification, separately of course, with 5 times their volume of water and then extracted with ether in the fashion recommended by Fischer and Abderhalden [12]. After evaporation of the ether strong hydrochloric acid was added to the residue and then the whole concentrated to dryness on the water-bath. Crystals obtained were thoroughly well washed and then converted by means of ammonia into the free amido acid; was recrystallised several times from water.

Analysis. 0·0560 grm. subst. gave 0·0356 grm. H_2O and 0·1345 grm. CO_2.
Found = 7·1 % H and 65·51 % C.
Calculated for $C_9H_{11}NO_2$ = 6·66 % H and 65·43 % C.

The watery solution of this fraction left after treatment with ether was saponified by means of baryta, the solution after removal of the barium with sulphuric acid was concentrated. The concentrated solution was then treated by Kutscher's [13] zinc oxide method for the separation of aspartic and glutamic acids, mainly with the view of obtaining the first named, but the result was not very satisfactory. In a preliminary digest which I had carried out with fibrin as substrate a fairly good yield of aspartic acid was obtained.

Fraction 125°—160° consisted mainly of glutamic acid and phenylalanine. After the removal of phenylalanine as above mentioned the fraction was saponified with baryta. After exact removal of the barium the filtrate was concentrated to a very small volume, and a large excess of strong hydrochloric acid added. After crystallisation was complete the crystals were filtered off at the pump, and thoroughly washed with strong HCl until they were quite white.

Analysis. 0·2636 grm. subst. gave 0·2079 grm. AgCl = 19·50 % Cl.
Calculated for $C_5H_9NO_4HCl$ = 19·34 %.
M.P. was 193°, thus agreeing closely with the 194° of Kutscher.

Conclusions.

The following substances have been definitely isolated from the products of digestion of coagulated blood serum with the lieno *a* protease of Hedin: histidine, inactive arginine, lysin, tyrosine, leucine, alanine, amido-valerianic acid, *a*-pyrrolidin carboxylic acid, glutamic acid, phenylalanine, and ammonia. Aspartic acid was also probably present;

it is formed in a large amount at any rate on digestion of fibrin by the same enzyme.

The most marked differences between the digestion carried out by Leathes and the present one are (1) the nature of the arginine [14] (Leathes had the optically active variety), and (2) in Leathes's digest there was an abundant yield of aspartic acid and little glutamic, whereas in the present case the yields are reversed. Neither Leathes nor I managed to isolate tryptophane, although we both obtained well-marked glyoxylic reactions.

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On the Physical Chemistry of the Toxin-Antitoxin Reaction: with Special Reference to the Neutralisation of Lysin by Antilysin.

By J. A. CRAW, Research Student, Lister Institute of Preventive Medicine.

(Communicated by Dr. C. J. Martin, F.R.S. Received March 22,—Read April 6, 1905.)

Ehrlich (1898) (1903) came to the conclusion that the toxin secreted by *B. diphtheriæ* is neutralised by diphtheria antitoxin much as an acid is by a base. The course of the neutralisation seems to him to indicate the presence of several toxic substances and atoxic forms of the same substances in the toxic fluid which are successively neutralised by the gradual addition of antitoxin. After complete neutralisation of the various toxins, a substance—toxone—remains which has the property of causing diphtheritic paralysis, and also of neutralising antitoxin. Similar constitutions have been ascribed by Ehrlich and his pupils to other toxic fluids, and to the hæmolytic filtrates or lysins obtained from certain bacterial cultures.

On the other hand, Arrhenius and Madsen (1902) (1904) concluded that the toxin-antitoxin reaction is quite analogous to the action of an acid on an alcohol, and that the chemical laws of mass action, which hold for the latter, apply equally well to the former. The chief reaction is considered to be a reversible one between two substances only, toxin and antitoxin, and when the system has reached equilibrium, a fraction of the toxin and also of the antitoxin remain free. The toxone effect they ascribe to a trace of free toxin. The numerical relations deduced are approximately in agreement with the experimental observations they have made on equilibria obtaining between toxins and antitoxins, and likewise between lysins and antilysins. Nernst (1904) has, however, pointed out that the laws of mass action are not applicable to these reactions.

Bordet (1903) expressed the view that the fixation of toxin by antitoxin is similar to the fixation of a dye by a tissue, and the author has shown that this conception is consonant with the chemical and physical properties of antibodies in general (1905).

The two substances most thoroughly investigated by Arrhenius and Madsen, viz., diphtheria toxin and tetanus lysin, do not admit of exact determination. The estimation of the free diphtheria toxin is rendered uncertain by reliance on animal experiments, and tetano-lysin is itself a most unstable body. Todd (1902), however, discovered a relatively stable lysin in the filtrate

from cultures of *B. megatherium*, and succeeded in producing a very strong antilysin for the same. He demonstrated that, in constitution, this lysin resembled tetano-lysin and diphtheria toxin. I, therefore, determined to test in how far the various views were applicable to the relations existing between Megatherium lysin and antilysin. Dr. G. Dean kindly placed the antilysin prepared by Dr. Todd at my disposal. I here beg to express my deep sense of indebtedness to Dr. G. Dean for his kindly criticism and many suggestions, and to Dr. C. J. Martin for his counsel in the filtration experiments.

On the hæmolytic index employed.—That the hæmolytic effect is always proportional to the amount of lysin added seems to be more than doubtful, consequently the calculated concentrations of free lysin have not been given.

However, when the hæmolytic powers of two solutions of the same lysin in the presence of a considerable concentration of antilysin are not widely different, the powers are approximately proportional to the amounts of free lysin present. The hæmolytic power of a fluid was determined by adding 1 c.c. to 2 c.c. of a 2·5-per-cent. suspension of washed guinea-pig corpuscles in 0·8-per-cent. saline, and heating the mixture to 37° C. for three hours.

The contents were subsequently allowed to stand in the ice-chest until the corpuscles had sedimented sufficiently to allow of 1 c.c. of the supernatant fluid being removed. The intensity of colour of the fluid was then compared with that of the tinted scale of a von Fleischl's hæmoglobinometer. By using a disc of paper stained with potassium chromate, instead of the usual white illumination, the contrast in colour between hæmoglobin and scale is much diminished, and daylight may be used for the comparison. Only that portion of the scale between the numbers 30 and 70 was used, and when the fluid was strongly coloured, dilutions were made so as to bring the final tint within these limits. The scale was standardised by various dilutions of the fluid obtained when 1 c.c. of lysin completely hæmolysed 2 c.c. of 2·5-per-cent. corpuscles in saline. The intensity of tint of this fluid has been represented by the index 100, and the hæmolytic indices given below refer to this tint as a standard. The experimental error in determining the tint was found to be less than 1 per cent. of the standard tint.

In the following a "partially neutralised" mixture of lysin and antilysin is a fluid giving a marked hæmolysis under standard conditions; a "neutral" mixture gives no hæmolysis in three hours, but would hæmolyse slightly in four hours; an "over-neutralised" mixture contains a greater proportion of antilysin than a neutral mixture.

On the presence of Free Lysin and Free Antilysin in mixtures which have attained equilibrium. Method: Filtration through Gelatine.

The method employed was that of C. J. Martin (1896), and applied by Martin and Cherry (1898), in their investigation of the relations existing between diphtheria toxin and antitoxin. The fluids examined were forced by a constant air-pressure of 100 atmospheres through Pasteur-Chamberland filters filled with solid gelatine, and each filtrate was removed in successive fractions of about 4 c.c. The hæmolytic powers of these fractions of filtrates, of the residual fluid left in the filter case, and of the original fluid introduced, were then determined in the manner described above.

Lysin.—On filtering a weak lysin solution through a filter prepared from 15-per-cent. gelatine, the filtrate gave little or no hæmolytic effect, but on diminishing the concentration of gelatine to 9 per cent., and using a stronger lysin, the filtrates obtained were strongly hæmolytic. The first few fractions had practically no action on blood corpuscles, and the succeeding fractions increased at first rapidly in hæmolytic power and then more slowly until a fairly constant value was reached, which approximated to that of the original lysin introduced. The gelatine, also, proved to be intensely hæmolytic, whereas the original gelatine had no effect on red-blood corpuscles. Candles prepared from 15-per-cent., 11-per-cent., 9-per-cent., and 7·5-per-cent. gelatine were also used, and the residual fluids showed in all cases a greater hæmolytic power than the original lysin, the difference being more marked with the higher percentages of gelatine. The gelatine filter is evidently more permeable to water than to the lysin. Similar concentration relations were found to hold for the filtration of crystalloids and inferior colloids, but the filter showed considerable differences in permeability to various crystalloidal substances.*

Antilysin.—On filtering a 5-per-cent. solution of the antilysin in saline no trace of anti-hæmolytic action could be detected in the filtrates. In this respect the antilysin behaves like a typical colloid, *e.g.*, colloidal ferric hydrate was found even in concentrated solutions to be almost entirely retained by the gelatine on filtering under *constant* pressure. It is important to observe that if the pressure be *suddenly diminished* the concentrated contents of the gelatine, whether crystalloidal or colloidal, are swept into the filtrate. The filter showed considerable permeability to typical colloids, especially those which stained the gelatine, but on the whole retained this class of substances to a greater extent than crystalloids.

* *Cf.* also E. W. Reid, 1901.

Mixtures.—The mixtures of lysin and antilysin were brought to a state of equilibrium by heating at 37° C. before filtering. They embraced weakly hæmolytic fluids (Nos. 1, 2, and 3, Table I), neutral fluids, *i.e.*, mixtures which did not hæmolyse in the standard time (No. 4), and fluids containing a large excess of antilysin (Nos. 5 and 6).

Nos. 1, 2, and 3 were exactly of the same constitution, viz., equal volumes of a lysin of constant value and a 5-per-cent. solution of antilysin in saline. No. 1 was heated 1 hour at 37° C., and kept at 10° C. for 1 hour. Nos. 2 and 3 were heated for 3 hours at 37° C., and allowed to stand 18 hours at 10° C.*

No. 4 consisted of equal volumes of a 1-per-cent. solution of a lysin which had been precipitated by ammonium sulphate, and of a 5-per-cent. solution of antilysin in saline. No. 5 contained one volume of the 1-per-cent. lysin to two volumes of 5-per-cent. antilysin, and No. 6, one volume of 1-per-cent. lysin to four volumes of 5-per-cent. antilysin. Nos. 4, 5, and 6 were heated for 3 hours at 37° C., cooled 1 hour at 10° C. and filtered.

Examination of the Filtrates for the Presence of Lysin.—The last fractions of the filtrate from No. 1 indicated a trace of hæmolysis, the corresponding fractions from Nos. 2 and 3 were unquestionably hæmolytic.

The filtrates from Nos. 4, 5, and 6, as well as the original fluids introduced, did not produce hæmolysis.

Examination of the Gelatine for the Presence of Lysin.—After filtration the *gelatine* of the filters 1 to 6 was melted out at 37° C., and *in all cases was found to be intensely hæmolytic*, whereas the original gelatine had no hæmolytic effect in the standard time.

Examination of the Residual Fluids for the Presence of Lysin.—The residual fluids were in all cases decidedly hæmolytic, as can be seen in Table I. *This increment in hæmolytic power was to be expected for Nos.* 1, 2, *and* 3 *from the results given above for the filtration of lysin alone*, and is simply a concentration effect that might be brought about by removing the water in other ways, *e.g.*, by evaporation under diminished pressure. The result, however, appears to be most remarkable when it is considered that No. 4 is a *neutral mixture*, and Nos. 5 and 6 *highly over-neutralised.*

Control filtrations of saline showed no hæmolytic power in either filtrate, residue, original fluid, or gelatine, so that the hæmolysis obtained above was certainly not due to impurities introduced by the apparatus.

* The experiment with No. 3 was performed 30 days after the experiments with Nos. 1 and 2 and the agreement in the hæmolytic values obtained testifies to the constancy of the lysin.

Table I.

Fluids mixed	Fluid lysin K_{XII}. Antilysin 5 per cent. in saline.						Precipitated lysin 1 per cent. in saline. Antilysin 5 per cent. in saline.							
No. of experiment	1		2		3		4		4A		5		6	
Volume ratio of lysin to antilysin	1 : 1		1 : 1		1 : 1		1 : 1		—		1 : 2		1 : 4	
Temperature and time of contact	37° 1 hr. 10° 1 hr.		37° 3 hrs. 10° 18 hrs.		37° 3 hrs. 12° 18 hrs.		37° 3 hrs. 10° 1 hr.		37° 2½ hrs. 10° 1 hr.		37° 3 hrs. 10° 1 hr.		37° 3 hrs. 10° 1 hr.	
	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.
Last fraction of filtrate	5	1·0	4	7·7	16	4·5	4	0	4	0	4	0	4	0
Total filtrate	75	0·5	63	6·3	72	2·8	54	0	62	0	48	0	35	0
Residue	40	38·6	35	41·3	37	38·2	33	31·2	33	27·8	42	21·6	15	20·1
Gelatine	—	100	—	100	—	100	—	100	—	100	—	100	—	100
Original mixture	120	16·8	105	24·3	112	16·8	98	0	100	0	100	0	62·5	0

The hæmolytic power of the residues and the gelatine could be demonstrated to be due to free megatherium lysin, for on the addition of sufficient antilysin *the hæmolytic power was in all cases entirely neutralised.* For example, 1 c.c. of residue No. 1 of index (38·6) with 1 c.c. of 5-per-cent. antilysin, added directly to the test blood, gave a hæmolytic index of (8·4), the same mixture heated for one hour at 37° C. before adding to the test blood, gave as index (1·8). Again, 0·5 c.c. of gelatine from No. 1 caused complete hæmolysis (index 100), whereas, after being heated for 1 hour at 37° C. with 1 c.c. of 5-per-cent. antilysin the value was reduced to (25). Free lysin, then, exists in the residues, and since the filtration was completed in less than two hours at a temperature of 10° C., at which temperature the velocity of reaction is extremely low, there can have been no appreciable liberation or dissociation of lysin during the filtration. It follows, therefore, that *free lysin exists in neutral and highly over-neutralised mixtures of lysin with antilysin, and that the free lysin is partially removed during filtration.*

The Existence of Free Antilysin in Partially neutralised and Neutral Mixtures.

The residual fluids from Experiments 1 to 6 showed slightly smaller hæmolytic indices after standing over night at 10° C., and on heating them to 37° C. for 1 hour their hæmolytic powers further markedly decreased. This is clearly shown by the following experiment:—

1 c.c. of residue No. 1 (index 38·6) together with 1 c.c. of saline, when added directly to the test blood, gave a hæmolytic index of (35), whereas the same mixture heated for one hour at 37° C. before being added to the test blood, gave an index of only (23·4). This behaviour indicates that in Nos. 5 and 6, the filtration which causes an increase in the concentration of any free lysin or antilysin by the withdrawal of water, occasions a further reaction which has a low velocity at 10° C., but is considerably more rapid at 37° C. As this phenomenon is common to all the residues it follows that if the above interpretation be correct, free antilysin is present in all cases, and presumably must have been present in the original mixtures. Nos. 1, 2, and 3 were, however, hæmolytic mixtures, and No. 4 neutral, which points to the conclusion that *free antilysin exists in partially neutralised and neutral mixtures* as well as in over-neutralised mixtures.

This conclusion is strengthened by the high neutralising power possessed by the original mixture and by the residue of No. 3, when fresh lysin was added, as shown by the following experiments:—

To one series of tubes containing 1 c.c. of the original hæmolytic mixture

in equilibrium, gradually increasing quantities of lysin were added. The same quantities of lysin were added to a second series containing 1 c.c. of the residue of No. 3, and to a third series containing 1 c.c. of saline. The test blood was added to each and, after mixing, the tubes were placed at 37° C. for 3 hours. The hæmolytic indices obtained are shown in Table II.

Table II.

Lysin K_{XII} added, in c.c.	Hæmolytic indices.				
	Saline.	Original mixture, No. 3.	Residual fluid, No. 3.	Original mixture increments.	Residual fluid increments.
0	0	25	48·2	—	—
0·0001	0	25	48·2	0	0
0·001	0	25	48·2	0	0
0·01	2·3	25	48·2	0	0
0·1	100	33·2	55·0	8·2	6·8
0·2	100	41·4	59·1	16·4	10·9
0·3	100	50·9	63·2	25·9	15·0
0·4	100	59·1	67·2	34·1	19·0

Obviously the original mixture has neutralised a very considerable proportion of the added lysin, for 0·1 c.c. of the lysin causes complete hæmolysis in saline (index 100), whereas 0·3 c.c. in the mixture only produces half the effect (index 50·9). The residue, although itself more hæmolytic than the original mixture, shows a higher neutralising value than the latter, as may be seen from the ratio of the increments, Table II. If the hæmolytic indices of mixtures containing a high concentration of antilysin such as those investigated above, are approximate measures of the free lysin present, then, for both original and residue, the amount of added lysin left free is roughly proportional to the lysin added. The amount of added lysin left free by the original is approximately double that left free by the residue, which agrees with the conclusion that the free antilysin in the latter is present in greater concentration than in the former.

On the Reversibility of the Reaction.—The reversibility of the reaction will be demonstrated if, on removing the free lysin, the compound of lysin with antilysin dissociates, producing more free lysin; on the other hand, if no lysin be set free, the reaction is irreversible. In the case of the reaction

being reversible, the velocity of dissociation will probably increase rapidly with rise of temperature. A temperature of 37° C. was employed as more likely to show reversibility if such exists.

The methods employed were: (*a*) filtration through gelatine; (*b*) diffusion through gelatine.

(a) *Filtration through Gelatine.*—The practically constant concentration of free lysin in the residues of Experiments Nos. 5 and 6, Table I, might well be due to reversibility. To test this the residue of No. 4 (index 31·25) was diluted with saline to the volume of the original mixture, and heated for $2\frac{1}{2}$ hours at 37° C. On again filtering through gelatine until the new residue had the same volume as the first residue, the hæmolytic index was found to be (27·8).* On repeating this treatment the hæmolytic index was found within the experimental error unchanged. The gelatine obtained from the filters used was strongly hæmolytic (index 100). If the free lysin of the first residue had alone been available, viz., $33 \times 31{\cdot}25 = 1031$ "hæmolytic units," then as filtration removes 300 or more "units," the second residue could not have had a higher index than $\frac{1031-300}{32{\cdot}5}$ $= (22{\cdot}5)$ and the third residue (13). It would appear then that a portion of the apparently combined lysin has become free on heating at 37° C.

I conclude, therefore, that *in a neutral mixture the reaction is at 37° C. at least partially reversible.*

(b) *Experiments on the Diffusion through Gelatine of Lysin and Antilysin.*—Dr. G. Dean kindly suggested to me a suspension of red blood corpuscles in gelatine as a convenient indication of the diffusion of lysin. In applying this method I found it was necessary to allow the lysin to diffuse from a solid gelatine layer into the solid gelatine suspension, for when normal saline alone is placed above a 2·5-per-cent. suspension of corpuscles in gelatine (9 per cent.), hæmolysis occurs. This proceeds from the surface downwards at a rate which is not altered when a strong fluid lysin is used instead of the saline. To avoid this effect, which is probably an imbibition phenomenon, the precipitated lysin was dissolved in 9-per-cent. gelatine at about 37°, and poured on to the strongly cooled columns of 9-per-cent. gelatine containing corpuscles. A rate of hæmolysis was then obtained which decreased with decreasing concentrations of lysin, and was entirely absent when saline in gelatine was used. The course of the diffusion is evidenced by a zone of transparent gelatine coloured with the freed hæmoglobin and a second zone in which partial hæmolysis has taken place, which

* *Cf.* Table I, No. 4A.

latter has a fairly sharp boundary against the unhæmolysed and turbid suspension. The experiments were carried out at about 18° C. The gelatine suspensions were contained in tubes of 1 cm. diameter, and were 10 cm. in length. The test volume of supernatant fluid was 1 c.c.

Lysin.—The rate at which hæmolysis proceeded from the surface decreased when the concentration of the lysin decreased from 1 to 0·0625 per cent., but lower concentrations down to 0·0078 per cent. gave almost the same effect as 0·0625 per cent. *For low concentrations, then, the rate of the hæmolytic effect is not dependent on the mass of the lysin.* Controls with saline gave no trace of hæmolysis.*

Antilysin.—A layer of the gelatine blood suspension 2 mm. thick was placed on a gelatine column containing 10 per cent. antilysin, and after standing for 40 hours at 18° C., a gelatine layer containing 0·5 per cent. lysin was superimposed. The blood was completely hæmolysed in 40 hours, whereas in a control with gelatine saline no hæmolysis took place. When, however, antilysin was mixed with the gelatine suspension, so that the concentration was 2·5 per cent., it was found that a gelatine layer containing 0·5 per cent. lysin failed to give a trace of hæmolysis in 40 hours. It follows, therefore, that the antilysin in the first experiment could not have been present in a concentration of 2·5 per cent. even in the layer immediately in contact with the 10-per-cent. antilysin in gelatine.

Again, as 0·5 per cent. lysin hæmolyses 2 mm. in 40 hours under normal conditions, no retardation has taken place, and it is probable that *megatherium antilysin does not diffuse appreciably through gelatine.*

Mixtures.—*A neutral mixture,* containing 0·5 per cent. precipitated lysin and 3·73 per cent. antilysin in saline gelatine (Table IV, No. 2), *and an over-neutralised mixture,* No. 1, containing 0·5 per cent. lysin and 5 per cent. antilysin, after being brought to equilibrium, *showed the presence of at least a trace of free lysin.* The hæmolytic effects were equal and of the magnitude for which

Table III.—Diffusion of Megatherium Lysin through Gelatine Columns, showing Hæmolysis, in Millimetres.

Percentage of lysin	1	0·5	0·25	0·125	0·0625	0·0317	0·0159	0·0078	Saline controls.
40 hrs.	2·6	2·0	1·7	1·4	1·0	1·0	1·0	1·0	0·0
70 „	4·5	3·5	2·2	1·5	1·0	1·0	1·0	1·0	0·0

* Compare Table III.

Table IV.—Diffusion of Lysin (0·5 Per Cent.) with Antilysin after attaining Equilibrium, through Gelatine Columns, showing Hæmolysis, in Millimetres.

Percentage of antilysin	No. 1. 5	No. 2. 3·73	No. 3. 2·5	No. 4. 1·25	No. 5. 0·625	No. 6. 0·317	Saline controls.
40 hrs.	tr.	tr.	tr.	tr.	1·4	2·0	0
70 ,,	1·0	1·0	1·0	1·0	1·5	3·5	0

no proportionality had been found to exist between mass of lysin and effect. It is remarkable that although in 70 hours this effect was evident, yet in less than 40 hours the corresponding free lysin had a greater hæmolytic effect. This seems most easily explained on Ehrlich's view that *lysins are complex and that antilysin will neutralise the most active constituents first.* The same effects were obtained from mixtures of lysin and antilysin in the same relative proportions, but present in $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$th of the concentration obtaining in the above experiment. Controls with saline showed no effect. The partially neutralised mixture containing 0·5 per cent. lysin and 2·5 per cent. antilysin No. 3, gave the same effect as the neutral mixture and likewise a mixture of 0·5 per cent. lysin and 1·25 per cent. antilysin, No. 4, but when the antilysin concentration was reduced to 0·625 per cent. (No. 5), the effect indicated a concentration equal to 0·125 per cent. free lysin. With 0·3175 per cent. antilysin (No. 6), and less the effect indicated 0·5 per cent. lysin free.

These results indicate that *the addition of antilysin up to* 1/10*th the amount required to entirely prevent hæmolysis* at 37° in the standard time (neutral mixture) *does not appreciably neutralise the lysin.* That the combination of lysin with antilysin is easily reversible at 18° C., does not seem probable, for on the addition of double the amount of antilysin the free lysin decreases to less than 1/4th of the total amount added. This then apparently conforms well with Ehrlich's view that no neutralisation of toxin takes place when small quantities of antitoxin are added.

On the Nature of the Equilibria: Fractional Addition of Lysin to Antilysin.

Danysz (1902) found that when a certain quantity of diphtheria toxin was added in fractions to antitoxin, less toxin was neutralised than in the case in which the whole quantity was added in one portion. This result has been confirmed by von Dungern (1904). Dr. C. Todd first drew my

attention to the fact that false equilibria were attained on the fractional addition of megatherium lysin to antilysin (experiments unpublished).

On further investigation I found, however, that the nature of the equilibria obtained in partially neutralised and in neutral mixtures is not the same. This may be seen from the following experiments :—

One c.c. of 0·625 per cent. antilysin was placed in each of two series of tubes. To one series 1 c.c. of various dilutions of lysin was added, and the mixture heated for two hours at 37° C. To the other series 0·5 c.c. of the same dilution of lysin was added, and the mixtures heated for one hour at 37° C. Another similar addition of 0·5 c.c. was then made, and the mixtures heated one hour at 37° C. Table V shows that the mixtures

Table V.

Addition at once.			Fractional addition.				
2 hrs. at 37°.			Part I, 1 hr. at 37°.		Part II, 1 hr. at 37°.		
Added to 1 c.c., 0·625 per cent. antilysin.					Added to Part I.		
Lysin in c.c.	Saline in c.c.	Hæm. index.	Lysin in c.c.	Saline in c.c.	Lysin in c.c.	Saline in c.c.	Hæm. index.
0·1	0·9	18·3	—	—	—	—	—
0·2	0·8	20·8	0·1	0·4	0·1	0·4	18·3
0·3	0·7	24·6	—	—	—	—	—
0·4	0·6	31·7	0·2	0·3	0·2	0·3	32·1
0·5	0·5	39·2	—	—	—	—	—
0·6	0·4	48·3	0·3	0·2	0·3	0·2	60·0
0·7	0·3	71·2	—	—	—	—	—
0·8	0·2	91·7	0·4	0·1	0·4	0·1	100
0·9	0·1	98	—	—	—	—	—
1·0	0·0	100	0·5	0·0	0·5	0·0	100

which were made in this intermittent way have higher hæmolytic indices than the mixtures which were made at one operation. The above was only found to be true when the resulting mixtures were strongly hæmolytic. When, however, the mixtures contained considerably less free lysin, the hæmolytic indices were the same in both series. From this and similar experiments, in which the volume of the antilysin was not appreciably modified by the addition of the lysin in concentrated form, it follows that false equilibria are attained in the lysin-antilysin reaction when the final concentration of the free lysin is high. In more nearly neutral mixtures

an apparently true equilibrium results. From Table V the irreversibility in nearly neutral solutions gives a slight effect in the opposite direction to the effect obtained by Danysz and von Dungern.

On the Mass Action of Lysin and Antilysin.

If the equation, toxin + antitoxin = 2 (toxin, antitoxin), proposed by Arrhenius and Madsen, holds for lysin and antilysin, then, when the same relative concentrations of these bodies are brought together, the equilibria attained will be the same, *i.e.*, the relative concentrations of the substances will be independent of the volume of the mixture. Thus, 10 c.c. of a mixture of lysin and antilysin in equilibrium will contain as much free lysin as 1 c.c. of a mixture 10 times as strong in total lysin and antilysin.

In Table VI (*a* 1, 2) and (*a* 5, 6) are such mixtures which were brought to equilibrium at 37°, the test volume (3) of the first mixture was 1 c.c., and of the other 0·1 c.c., added with 0·9 c.c. saline (7). The hæmolytic indices (4) (8) are not equal, being respectively (50) and (60).

Table VI.—Hæmolytic Indices of Multiple Mixtures of Lysin and Antilysin in Equilibrium.

	1.	2.	3.	4.	5.	6.	7.	8.
	Lysin conc. per c.c.	Antilysin conc. per c.c.	Test volume in c.c.	Hæm. index.	Lysin conc. per c.c.	Antilysin conc. per c.c.	Test volume in 1 c.c.	Hæm. index.
a	0·005	0·01	1·0	50	0·05	0·1	0·1	60
b	0·0025	0·005	1·0	26	0·025	0·05	0·1	50
c	0·0005	0·001	1·0	0	0·05	0·1	0·01	42·5
d	0·00025	0·0005	1·0	0	0·025	0·05	0·01	41

Two similarly related mixtures of half the strength (*b* 1, 2) and (*b* 5, 6) showed a more marked difference, viz. (26) and (50). When the strength of the mixtures were widely different as 1—100, the difference is extremely marked, *e.g.*, (*c* 1, 2) and (*c* 5, 6) gave the indices (0) and (42·5) when 1 c.c. and 0·01 c.c. with 0·99 c.c. saline were used as the test volume respectively. Two similarly related mixtures of half the strength (*d* 1, 2) and (*d* 5, 6) showed a similar effect and indices (0) and (41).

It must then be concluded that Arrhenius and Madsen's equation does not apply to the lysin-antilysin reaction so far as megatherium lysin is concerned.

The Validity of the Application of Chemical Mass Action Equations to the Lysin-Antilysin Action.

Nernst (1904) has insisted that the views of Arrhenius and Madsen on the mass action of toxin and antitoxin can have no true theoretical foundation. If the reaction consist in a chemical change, it seems to me that the chemical law of mass action would be applicable in the manner given below. If, on the other hand, the observed mass action differs widely from the deduced, we can conclude that the toxin-antitoxin reaction is not a purely chemical one.

The anti-body, *e.g.*, antilysin, is a typical colloid, and its active chemical mass will, like that of a solid suspension, be constant. Similarly, the chemically active mass of the compound between antilysin and lysin is constant, as this also occurs in the form of a fine suspension. The active mass of the lysin will vary with its concentration, as this substance is not present in the form of a typical colloid. It has properties such as diffusibility, and the power of passing a gelatine filter similar in magnitude to those found for crystalloids and inferior colloids.

These conditions are not unlike those studied by Walker and Appleyard (1896) in the case of a solid suspension of diphenylamine in picric acid solutions. If this analogy is a true one, the chemical equation expressing the combination of lysin with antilysin should be similar to that given by these authors for the combination of picric acid with diphenylamine, viz., lysin, water + antilysin = compound + water.

The lysin is removed by the solid suspension of antilysin, and a solid suspension of compound (lysin, antilysin) is produced with the liberation of the water which held the lysin in solution. The velocity of combination will be K_1CK' where K_1 is the velocity constant, C the concentration of the lysin, and K' the constant active mass of the antilysin. When the reaction is reversible, *i.e.*, possibly at high concentrations of antilysin, the velocity of dissociation will be $K_2K''K'''$ where K_2 is the velocity constant, K'' the constant active mass of the compound, and K''' the constant active mass of the water. For equilibrium $K_1CK' = K_2K''K'''$, or

$$C = \frac{K_2K''K'''}{K_1K'} = \text{constant.}$$

From which it follows that at any definite temperature the concentration of the lysin must be a constant. The compound (lysin, antilysin) and free antilysin can only exist side by side in the aqueous medium when the latter has a certain fixed absolute concentration. The relative concentrations of

free antilysin and compound have no influence on the equilibrium. Thus Walker and Appleyard found that the concentration of aqueous picric acid in contact with varying amounts of diphenylamin is constant, but that the latter was stained more deeply when present in smaller quantities. The relations observed between lysin and antilysin are, however, totally different, for in this case the amount combined varies continuously with the concentration of the lysin.

I therefore conclude that *the removal of lysin from a solution by antilysin is not capable of interpretation as a purely chemical change, and the law of chemical mass action does not apply when the lysin is present in excess.*

From the filtration experiments with neutral and overneutralised mixtures it, however, seems possible that when excess of antilysin is present, the chemical law of mass action holds, for the concentration of free lysin was found to be practically constant when the concentration of antilysin was largely increased.

The application of Adsorption and Surface Tension hypotheses to the Lysin-Antilysin Action.

Walker and Appleyard also investigated the phenomena of the fixation of picric acid by silk, to which the relations of lysin to antilysin, which have been described above, present a much closer analogy. They found that the concentration of the dye bath varies continuously with the depth to which the silk is dyed, and that the concentration of picric acid (C_2) in the silk was proportional to the concentration of free picric acid (C_1) raised to a constant power n, or $C_2 = KC_1^n$. The adsorption of substances, *e.g.*, of iodine from solutions by charcoal (Schmidt, 1894); of iodine from water by starch (Küster, 1894), etc., have in general been found to obey similar relations. It is, then, not improbable that some such adsorption formula may be found to hold for the fixation of lysins, etc., by their respective antibodies, as I suggested in a recent paper on the phenomena of agglutination (1905). I am at present engaged in further investigating the phenomena of lysin-antilysin relations from this point of view, and, so far, the results have been encouraging.

Summary of Conclusions.

1. Megatherium lysin passed through a gelatine filter, and is diffusible through gelatine.

2. Megatherium antilysin does not pass through a gelatine filter, and is not appreciably diffusible through gelatine.

3. The filtration and diffusion of mixtures show that free lysin is present in neutral mixtures and in mixtures containing excess of antilysin.

4. Free antilysin exists in neutral mixtures, and in mixtures containing excess of lysin.

5. The reaction is at least partially reversible when excess of antilysin is present.

6. False equilibria are produced with greater facility when the lysin is in excess.

7. The neutralisation equation of Arrhenius and Madsen does not hold for multiple mixtures.

8. The removal of lysin from a solution by antilysin is not capable of interpretation as a purely chemical change, but is more analogous to certain adsorption phenomena.

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Journal Physiology, Vol. XXXII.

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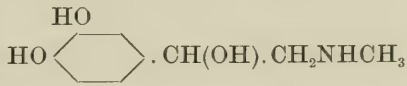
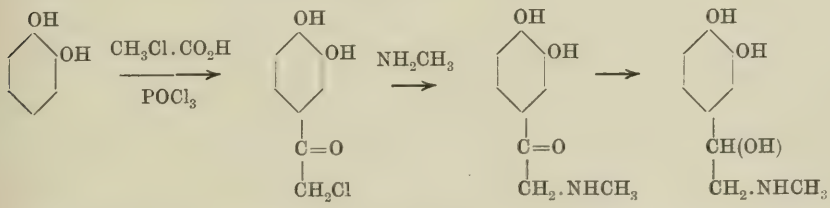
The physiological action of synthetical substances allied to adrenalin. By H. D. Dakin.

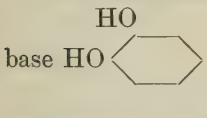
The results of the combined researches of von Fürth, Jowett and Pauly have led to a fairly exact knowledge of the chemical structure of adrenalin. The formula which has received the most support is as follows:



Starting from pyrocatechin it is possible to prepare a base whose chemical and physiological properties are very similar to those of adrenalin. The changes are outlined below, the chemical details being reserved for a future communication.



The formula of the synthetic base is given provisionally, subject to further investigation. The salts of the base show all the chemical reactions of natural adrenalin, but certain differences appear to exist between the free bases. Whether this is due to the fact that the synthetic base is optically inactive whereas the natural substance is active is not yet clear. The physiological activity of the new substance is about the same as adrenalin. Less than one millionth of a grm. produces a definite rise in blood-pressure on injection into a rabbit.

An intermediate product in the synthesis described is the ketone base 

This base has also been obtained by Stolz and by Friedmann and its physiological properties have been

investigated by Hans Meyer. Its action in producing a rise in blood-pressure is however insignificant when compared with that of its reduction product, and in fact is not much more powerful than the chloracetylpyrocatechin from which it is prepared. A large number of allied ketone bases has been prepared and an attempt has been made to connect their chemical structure and physiological action. From a chemical point of view they may be divided into the following classes:

I. The simplest member of the group $C_6H_3(OH)_2.\underset{\underset{O}{\|}}{C}.CH_2NH_2$.

II. Derivates of the type $C_6H_3(OH)_2.\underset{\underset{O}{\|}}{C}.CH_2NHR$.

(*a*) Where R is an aliphatic group, *e.g.* methyl, ethyl, amyl, and heptyl.

(*b*) Where R is a mixed group, *e.g.* benzyl.

(*c*) Where R is purely aromatic, *e.g.* phenyl, tolyl, napthyl.

III. Derivatives of the type $C_6H_3(OH)_2.\underset{\underset{O}{\|}}{C}.CH_2.N\begin{matrix}R\\R\end{matrix}$ *e.g.* di-methyl, di-amyl.

IV. Derivatives of the type

$$C_6H_3.(OH)_2.\underset{\underset{O}{\|}}{C}.CH_2.N\begin{matrix}R\\R\\R\\OH\end{matrix}$$ *e.g.* tri-methyl, di-methyl-phenyl.

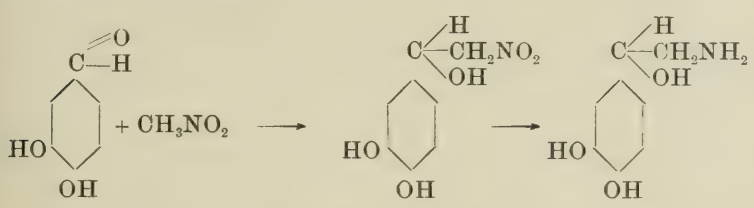
The physiological action of these bases, as regards the blood-pressure effect, bears a very distinct relation to their structure. Substances in Classes I and II(*a*) all produce a marked rise in blood-pressure in doses of about 1 milligram per kilo and on reduction yield bases whose activity is comparable with that of adrenalin. The substance representative of Class II(*b*) stands in both chemical and physiological respects between II(*a*) and II(*c*). The members of the latter class cause a fall of blood-pressure which may be followed by a slight rise. In some cases at least the injection of their reduction products causes a very marked rise in pressure but the action is not nearly so powerful as in the case of the aliphatic bases.

The physiological action of the di-alkyl bases (Class III) appears to be similar but less marked than the mono-alkyl bases. Their reduction products are however very active. Only two substances of Class IV have

so far been examined—the tri-methyl compound was found to be very active whilst the aromatic derivative with the phenyl-di-methyl groups was inactive.

Injection of pyrocatechin itself, in doses of about two milligrams per kilo produces a very distinct rise in blood-pressure, and this is also true of many of its simple derivatives and homologues, *e.g.* protocatechuic aldehyde, 3·4 di-oxyacetophenone, chloracetylpyrocatechin, 3·4 di-oxyphenylmethylcarbinol, pyrogallol, chloracetylpyrogallol and alkylamino-acetylpyrogallols. Substances in which the hydrogen of the phenol groups is replaced appear to be practically inactive.

A further attempt at the synthesis of the simplest member of the groups of substances to which adrenalin is supposed to belong, was made on the following lines. It is possible to condense protocatechuic aldehyde with nitromethane without the elimination of water. On reduction the nitro-group may be converted into an amido-group. The changes may be represented as follows:



The base formed in this reaction was found to be physiologically active.

A detailed account of the preparation and properties of the substances mentioned will be published shortly.

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THE FRACTIONAL HYDROLYSIS OF OPTICALLY INACTIVE ESTERS BY LIPASE. PART II.

By H. D. DAKIN.

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In previous communications[1], it was shown that if an optically inactive ester such as methyl-mandelate be partially hydrolysed by the enzyme lipase, the mandelic acid liberated is strongly dextro-rotatory whilst the residual ester is correspondingly laevo-rotatory. The mandelic acid resulting from complete hydrolysis is of course completely inactive and may be almost quantitatively recovered. It is thus clear that the dextro component of the ester is hydrolysed more rapidly than the laevo component, and this necessitates the conclusion that the hydrolysis is indirect, for the production of optically active products requires the intervention of some asymmetric substance.

It is well known that in ordinary chemical reactions when optical isomerides separately combine with the same structurally asymmetrical substance they may do so with unequal velocity, and conversely the products formed by such reaction, since they are no longer optical opposites, may also undergo further change at unequal rates[2]. By analogy, it was therefore concluded that the enzyme lipase—or conceivably some substance intimately associated with it—was a powerfully optically active substance and that definite chemical union took place between it and the ester undergoing change. If the ester contained an asymmetric carbon atom, the additive compounds formed between the dextro and laevo components of the ester and the enzyme would not be optical antipodes, and therefore the rate of combination of the enzyme with the two optical components may be different, and further the rate of decomposition of the two additive compounds may again be unequal and thus account for the production of optically active products as a result of partial hydrolysis.

For example, if the enzyme (e) be assumed, for purposes of illustration,

[1] *Proc. Chem. Soc.* XIX. p. 161. 1903; *Journ. Phys.* XXX. p. 253. 1903.

[2] Cp. *Trans. Chem. Soc.* LXXXV. p. 1514. 1904.

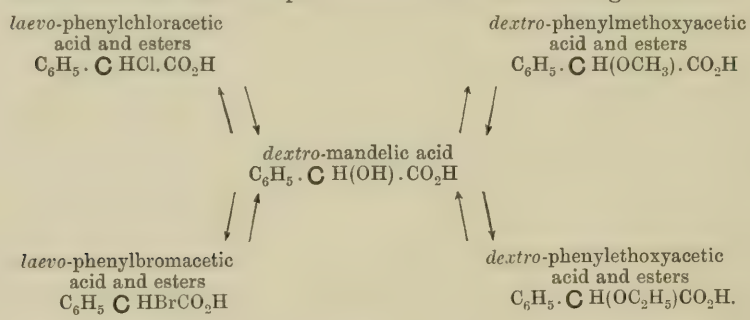
to be dextro-rotatory and the two components of the inactive ester be represented by $+S$ and $-S$, the two additive compounds formed by union of the ester and enzyme would be $(+e, +S)$ and $(+e, -S)$. These two compounds are clearly not optical opposites and therefore may be formed and undergo change at different rates. The optical opposite of the first complex would obviously be $(-e, -S)$, and of the second $(-e, +S)$.

In the previous communications the hydrolysis by means of lipase of the methyl, ethyl, *iso*-amyl, and benzyl esters of mandelic acid was studied[1]. The investigation has now been extended in two directions:

(*a*) The hydrolysis of esters structurally allied to mandelic ester.

If, as is assumed, the course of the hydrolysis of inactive esters by lipase is influenced by the configuration of the complex molecules (enzyme + *dextro* or *laevo* ester) one would expect that the hydrolysis of closely related esters would give in the main similar products. Thus, for example, the partial hydrolysis of all the esters of mandelic acid so far examined has yielded a dextro-rotatory free acid together with a residue of laevo-rotatory ester. Starting from active mandelic acid it is possible to prepare two sets of derivatives both of which possess a "similar configuration" but which differ in sign of rotation. Thus *dextro*-mandelic acid gives on the one hand *dextro*-phenylethoxyacetic acid but *laevo*-phenylchloracetic acid on the other, and both derivatives may be reconverted into dextro-mandelic acid[2].

Some of these relationships are shown in the following scheme :



[1] Fischer and Bergell (*Ber. d. Deutsch. chem. Ges.* XXXIII. p. 2592, 1903) have recently described a reaction which appears to resemble the selective hydrolysis of optically inactive esters. Carbethoxyglycyl-leucine, prepared by condensing carbethoxyglycine with racemic leucine, was digested with trypsin. After digestion the solution became dextro-rotatory and contained an oil which was probably carbethoxyglycyl-d-leucine together with leucine which was apparently a mixture of the laevo and inactive acids. The mechanism of the hydrolysis of polypeptides by trypsin and of esters by lipase may therefore be essentially similar.

[2] Cp. Walden, *Ber. d. Deutsch. chem. Ges.* XXVIII. p. 1295. 1895; McKenzie, *Trans. Chem. Soc.* LXXV. p. 753. 1899.

It was therefore anticipated that the partial hydrolysis by lipase of esters of phenylethoxyacetic acid and of phenylchloracetic acid would yield a dextro-rotatory acid in the former case but a laevo-rotatory acid in the latter. This was found to be so and like results were obtained in several other similar cases. The main results are shown in the following table:

Ester partially hydrolysed by Lipase	Sign of Rotation of: Acid liberated	Sign of Rotation of: Residual ester
Methyl-mandelate	+	
Ethyl ,,	+	−
Iso-amyl ,,	+	−
Benzyl ,,	+	−
Methyl-phenylchloracetate	−	+
Methyl-phenylbromacetate	− (?)	+
Ethyl-phenylchloracetate	−	+
Ethyl-phenylbromacetate	− (?)	+
Methyl-phenylmethoxyacetate	+	−
Ethyl-phenylethoxyacetate	+	−

It therefore appears that in the fractional hydrolysis of a series of allied inactive esters, the components which are most readily attacked possess a similar configuration but not necessarily a similar sign of rotation. In this connection the fact may be recalled that when a series of inactive acids is resolved by means of the biological method, using the same organism throughout, the active products obtained generally have a similar configuration[1].

(*b*) The hydrolysis of inactive esters in which an asymmetric carbon atom is present in the alkyl group.

It might be suggested that the production of optically active substances in the reactions under consideration might not be due to a combination of the enzyme with the ester as a whole, but to the combination of the enzyme with the acid liberated by the decomposition of the ester. Although there are no grounds for assuming that the latter reaction does not take place, yet it appears certain that both alkyl and acyl groups—and hence probably the ester molecule as a whole—combine with the enzyme, since a selective hydrolysis is observable irrespective of whether the asymmetric carbon atom is situated in the acyl or alkyl group. Thus, for example, the products of the partial hydrolysis by lipase of the acetate of phenylethylcarbinol $\left(\begin{matrix}C_6H_5\\C_2H_5\end{matrix}\!\!>\!C<\!\!\begin{matrix}O.CO.CH_3\\H\end{matrix}\right)$ were distinctly laevo-rotatory.

[1] Harden and McKenzie. *Trans. Chem. Soc.* LXXXIII. p. 424. 1903.

A few experiments on the hydrolysis of aliphatic esters by lipase have not yielded nearly such positive results as the aromatic esters have given. So far di-ethyl aspartate, ethyl bromisovalerate and methyl racemate have been hydrolysed. The practical difficulties of working with the aliphatic esters is much increased by their comparatively small optical rotations, and in addition it may well be expected that the two optical components of the inactive esters of this class would not show such a different rate of hydrolysis as is the case with the aromatic esters.

Experimental.

The lipase solution used in the following experiments was prepared from pig's liver. The fresh organ was minced, mixed with kieselguhr and the juice obtained by means of an hydraulic press. The liquid thus obtained is rich in lipase and may be preserved for several weeks if kept on ice. The liquid is faintly acid and the acidity slowly increases with age. Owing to the extremely unstable nature of the enzyme it has not yet been found possible to even roughly purify the ferment, but special blank experiments showed that under the existing conditions, the products of hydrolysis isolated by the method to be described did not contain impurities sufficient to show a measurable rotation.

In the majority of experiments, the liver-juice was diluted about twenty times, and the resulting solution was vigorously shaken with one to two grams of the ester to be hydrolysed. It has not been found practicable to work with the much larger amounts of substance which one would by preference employ, partly, I believe, on account of the "poisoning" action of the esters. A small quantity of chloroform was usually added as antiseptic, but care had to be taken to avoid excess as otherwise the bulk of the ester was withdrawn from the sphere of action of the enzyme. Control experiments were always carried out in which the enzyme was destroyed by boiling before the ester was added. It was found convenient to carry out the reactions at about 20°. Hydrolysis proceeded more quickly at a higher temperature, but on the other hand the enzyme was more rapidly destroyed, so that no ultimate advantage was obtained.

It was found essential to shake the mixture of enzyme solution and ester very vigorously, for otherwise, at least in the case of the sparingly soluble esters, very little hydrolysis took place. Within a short time of

the commencement of the experiment a proteid precipitate was usually thrown out of solution owing to the accumulation of free acid and this was of great assistance in keeping the ester in a finely divided condition. The reaction was usually allowed to proceed for 12—36 hours and the amount of hydrolysis was determined by titrating with standard alkali aliquot parts of the solutions from both the actual and control experiments. The products of hydrolysis were usually extracted with chloroform or ether as solvents, and after separation into acid and ester fractions their alcoholic solutions were examined in the polarimeter. It must be clearly understood that the rotations measured are merely recorded to show whether a distinct resolution into active substances has been effected or not. The experimental difficulties of isolating such small quantities of esters and acids from such a complex enzyme solution are so great as to render the results of little quantitative value. The difficulty of complete extraction of the substances from solutions containing proteids is very great, and further it is extremely difficult to avoid further hydrolysis of the ester remaining unattacked by the enzyme.

Preparation and hydrolysis of methyl-phenylchloracetate. Phosphorus pentachloride (1 mol.) and mandelic acid (1 mol.) were heated together at a temperature gradually rising from 70–130°. The resulting liquid was fractionated *in vacuo.* The portion boiling at 120–140° is somewhat impure phenylchloracetyl chloride. On boiling this with methyl alcohol and purifying the product in the usual way, pure methyl phenylchloracetate boiling at 129–130° under 15 mm. pressure is readily obtained. The yield is about seventy per cent. of the weight of mandelic acid employed.

Two grams of the ester were added to 250 c.c. of 5 % lipase solution and hydrolysis was allowed to proceed until about 37 % of the ester was decomposed. The liquid was then extracted with ether in a continuous extractor. The ethereal solution containing both the unchanged ester and acid was shaken with 5 c.c. normal sodium carbonate solution and the still alkaline liquid at once separated and acidified with sulphuric acid. The acid liquid was then re-extracted with ether, the ether removed and the residue dissolved in absolute alcohol. The solution was then clarified by shaking with charcoal, filtered and examined in the polarimeter. A rotation of $-\cdot 51°$ was observed. The ethereal solution containing the unchanged ester was concentrated, and the alcoholic solution showed a rotation of $+1\cdot 01°$.

Ethyl phenylchloracetate. This substance was prepared in the same

way as the methyl ester by boiling phenylchloracetyl chloride with ethyl alcohol. 1·5 grams of the ester were shaken with 250 c.c. of 5 % lipase solution until about 50 % had been hydrolysed. The unchanged ester, isolated as in the previous case, had a rotation of + ·42°. The phenylchloracetic acid was neutralized by soda and showed in aqueous solution a rotation of − ·22°.

Preparation and hydrolysis of methyl phenylbromacetate. The preparation of this substance in the pure state gave some little difficulty, but eventually a convenient method was obtained. The reaction between mandelic acid and phosphorus pentabromide is not nearly so smooth as is the case with pentachloride[1], and the impure phenylbromacetylbromide yielded on treatment with methyl alcohol an ester which boiled at too low a temperature. Christomanos[2] has shown that phosphorus and bromide react quietly when brought together in the presence of benzene, and this fact was made use of in the present case. Moreover instead of acting upon mandelic acid and obtaining the acid bromide it was found far better to act upon methyl mandelate with phosphorus tribromide. The details are as follows : phosphorus (1 mol.) is well covered with dry benzene and bromide (3 mols.) added drop by drop with cooling. The reaction is quite moderate and free from danger. Methyl mandelate (3 mols.) dissolved in benzene is then added by degrees. A vigorous reaction takes place which is completed by heating upon the water-bath for some time. The cooled liquid is then cautiously washed with water, dried over calcium chloride and fractionated. Methyl phenylbromacetate distils at 155–157° under 26 mm. pressure and the yield is equal in weight to the methyl mandelate taken.

Two grams of the ester were vigorously shaken with 250 c.c. of 5 % lipase solution till slightly over 50 % had been hydrolysed. After extraction the unchanged ester had a rotation of + 1·52°. The free acid, after separation, was practically inactive, this being in all probability due to its being decomposed by the treatment with alkali, since the sodium salt of phenylbromacetic acid is extremely unstable.

Ethyl phenylbromacetate. This substance was prepared in the same way as the methyl ester by acting upon ethyl mandelate with phosphorus tribromide in benzene solution. The vapour of both esters is extremely irritating. One gram of the ester was dissolved in a little spirit and then well shaken with 200 c.c. of 2½ % lipase solution until about sixty per cent. of the ester had been decomposed. The unchanged

[1] Cp. Hell and Weinzweig. *Ber. d. Deutsch. chem. Ges.* XXVIII. p. 2445. 1895.

[2] *Ber. d. Deutsch. chem. Ges.* XXXVII. p. 2883. 1903.

ester on subsequent extraction with ether showed a rotation of $+\cdot 50°$, whilst the free acid, as in the case of the methyl ester, was almost inactive.

Methyl phenylmethoxyacetate. This ester was prepared by the action of silver oxide and methyl iodide upon mandelic acid[1], one gram of the ester was hydrolysed to the extent of twenty-five per cent. by shaking with 200 c.c. of lipase solution. The recovered ester was laevo-rotatory $-\cdot 22°$, whilst the free acid had a rotation of $+\cdot 26°$.

Ethyl phenylethoxyacetate. This ester was prepared according to M^c^Kenzie's directions (*loc. cit.*). One gram was shaken with 200 c.c. of lipase solution until about seventy per cent. had been decomposed. The unchanged ester was laevo-rotatory whilst the free acid showed a dextro-rotation of $+ 1\cdot 18°$.

Phenylethylcarbinol acetic ester. Phenylethylcarbinol was prepared according to Grignard's directions by acting upon benzaldehyde with magnesium ethyl iodide. The acetic ester of this alcohol is not very readily attacked by lipase, and the difficulties of the experiment were increased by the fact that no simple method for the separation of the small quantities of alcohol and unchanged ester could be found. However, in one experiment, two grams of the ester were hydrolysed to the extent of twenty-five per cent. by 300 c.c. of 5 % lipase solution, and on extracting the liquid with chloroform the mixed products of the reaction were found to show a laevo rotation of $-\cdot 31°$. Whether this rotation is due to phenylethylcarbinol or to its ester is not yet clear, but the result is sufficient to show that a selective hydrolysis had taken place.

Summary.

(1) The partial hydrolysis of the inactive esters of mandelic acid and its alkyl derivatives results in the production of a dextro-rotatory free acid and a laevo-rotatory residue of unchanged ester. In the case of the derivatives of mandelic ester in which the hydroxyl group is replaced by a halogen atom a laevo-rotatory free acid is formed together with a dextro-rotatory residue of ester. The results are in agreement with the explanation offered in a previous paper, and further show that when a series of allied optically inactive esters is hydrolysed by lipase the active components which are most readily attacked possess a

[1] M^c^Kenzie. *Trans. Chem. Soc.* LXXV. p. 753. 1899.

similar molecular configuration but not necessarily the same sign of rotation.

(2) The partial hydrolysis of an optically inactive ester, in which the asymmetric carbon atom was situated in the alkyl portion of the molecule, instead of in the acyl group as in the other esters examined, also yielded optically active products, showing that the enzyme combined both with the acyl and alkyl portions of the ester molecule and hence presumably with the ester molecule as a whole.

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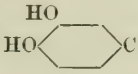
On the Physiological Activity of Substances Indirectly Related to Adrenalin.

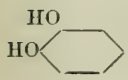
By H. D. DAKIN.

(Communicated by Professor E. H. Starling, F.R.S. Received May 29,—Read June 8, 1905.)

(From the Laboratory of Pathological Chemistry, Lister Institute of Preventive Medicine.)

Since adrenalin itself possesses such remarkably definite physiological properties which are shared by the synthetical substance described in the preceding paper, it seemed to be of interest to try and trace some connection between their chemical structure and physiological action and, in particular, to see if the activity was to be ascribed to any particular chemical group or combination of groups.

If the formula for adrenalin be either 

or 

, we may artificially divide the molecule into two portions, an aromatic catechol nucleus and an oxyethylmethylamine ($CH_2OH.CH_2NHCH_3$) side-chain. The latter substance was prepared by heating glycolchlorhydrin with methylamine (1) and it was found that injection into a rabbit of quantities up to 10 milligrammes was followed by slight if any rise in blood-pressure. Injections of catechol, on the other hand, produce a great increase in pressure, as may be seen in fig. 1. This fact has also been observed by Mühlmann (2). The amount of substance required to produce an effect is considerable when compared with adrenalin, but a positive result seems significant. It will be seen from the tracing that the effect is prolonged, and it was found that subsequent injection of adrenalin produced no higher retinal pressure than that caused by catechol. When smaller quantities are injected into rabbits it is found that one does not obtain a rapid rise followed by a rapid fall, but both phases are prolonged and the stimulation is sub-maximal.

The power of causing increased blood-pressure is shared by many other substances containing the catechol nucleus. Thus the two intermediate products in the synthesis of the "adrenalin-like" base described in the preceding paper are both physiologically active. In the case of chloracetyl-

catechol several milligrammes are required to produce well-marked effects, but methylamino-acetylcatechol is more nearly related to adrenalin, and, as one would expect, is more active physiologically. The properties of this substance have been investigated by Hans Meyer (3) so that it is unnecessary to give further details, but it may be noted that about half a milligramme is necessary to produce a definite rise in blood-pressure in a rabbit, so that the activity of the substance is far inferior to that of adrenalin.

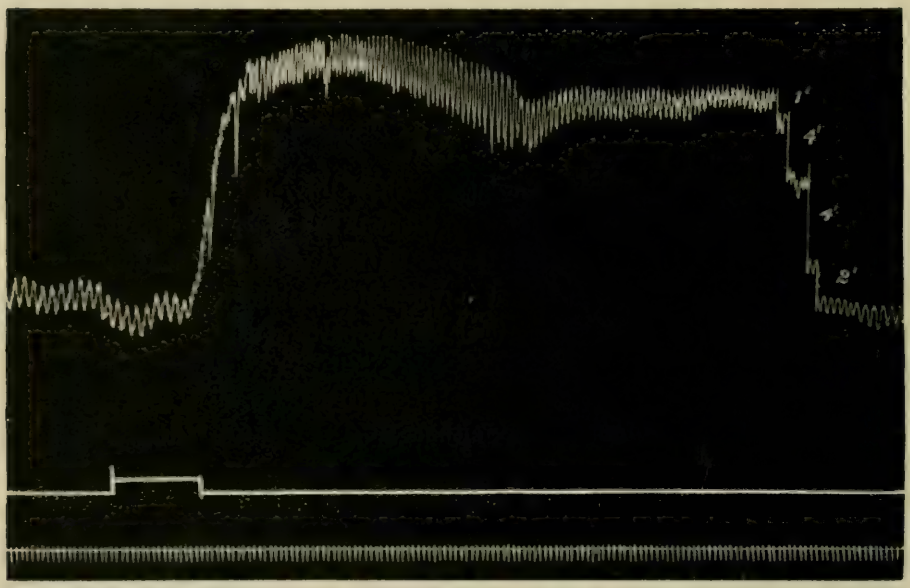


FIG. 1.—Rabbit, 2·2 kilogrammes. Carotid B. P. Vagi divided. Urethane. 10 milligrammes catechol. Zero pressure 30 mm. below signal line. Time = seconds.

If the chlorine in chloracetylcatechol be replaced by hydrogen the product acetylcatechol, $C_6H_3(OH)_2.CO.CH_3$, is still active, but if the hydrogen of the hydroxyl groups be replaced, for example, by acetyl groups the product is quite inactive. Similarly, although, as previously stated, injection of catechol is followed by increase in blood-pressures, the methyl ether of catechol, $CH_3.O.C_6H_4(OH)$, produces no such effect and other analogous cases have been observed. It may be noted that substitution of the hydrogen of the phenolic hydroxyl groups very greatly increases the chemical stability of these substances and this, one may well imagine, would tend to result in substances of less marked physiological activity. From these results it would appear that two free hydroxyl groups in the nucleus are essential constituents of active substances in this group and, since of the three isomeric dihydroxybenzenes only catechol produces a rise in blood-pressure after injection, it is possible that the hydroxyl groups must be in the *ortho* position to one

another. Further work is needed, however, before the question can be definitely decided.

As has been already stated, methylamino-acetylcatechol is fairly active in causing increased blood-pressure, whilst the activity of its reduction product is comparable with that of adrenalin. It seemed to be of interest to try the action of the amino- and other alkylamino-acetylcatechols and their reduction products. A considerable difference is noticeable in their physiological properties, corresponding to differences in chemical structure. Thus, amino-acetylcatechol ($C_6H_3(OH)_2.CO.CH_2NH_2$), and the lower alkylamino-acetylcatechols, *e.g.*, the ethyl and di-methyl derivatives, closely resemble the methylamino-acetylcatechol previously described, and their reduction products are very active.

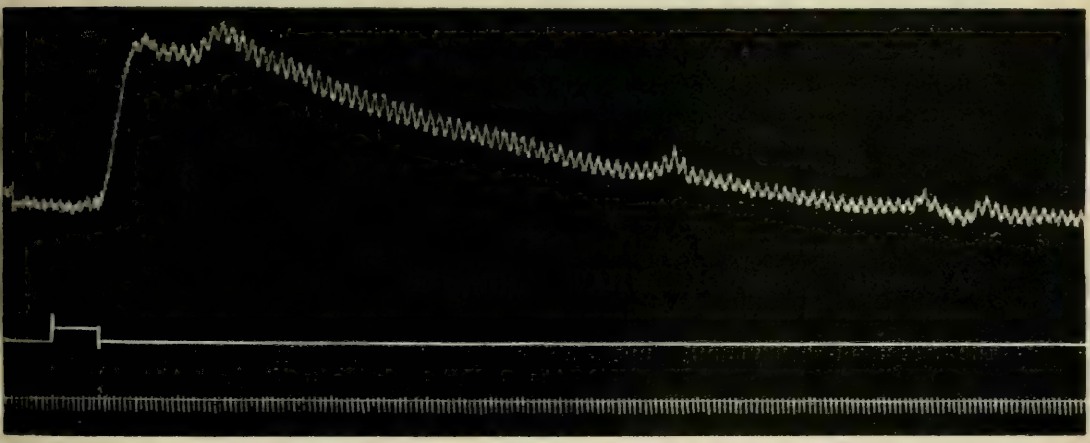
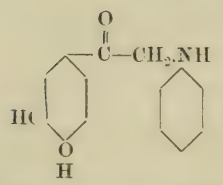


FIG. 2.—Rabbit, 2·5 kilogrammes. Carotid B. P. Vagi divided. Urethane. 0·0010 gramme hydrochloride of ethylamino-acetylcatechol. Zero pressure 30 mm. below signal line. Time = seconds.

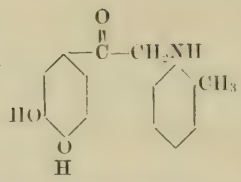
If, however, one takes a higher member of the same series, *e.g.*, heptylamino-acetylcatechol, $C_6H_3(OH)_2.CO.CH_2NH(CH_2)_6.CH_3$, it is found that whilst the ketone base is still active, yet on reduction its activity is but slightly increased.

The base was prepared by the action of excess of heptylamine upon the di-acetyl derivative of chloracetylcatechol, and is a white crystalline substance melting at 125°, and giving beautifully crystalline salts.

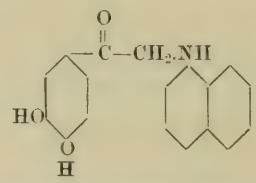
This result shows that the nature of the alkyl group attached to the nitrogen atom is of great importance. If, instead of an aliphatic one substitutes an aromatic group attached to the nitrogen atom the changes in physiological properties are very marked. The following bases were examined :—



Anilino-acetylcatechol (4).



o-Toluidino-acetylcatechol (4).



α-Naphthylamino-acetylcatechol.

None of these bases produced a decided rise in blood-pressure on injection of small quantities. Usually, a fall in pressure was noted, which was least marked with the first substance, and was in this case sometimes followed by a slight rise. This rise was more marked when the substance obtained by its reduction was injected, but even then its activity was far behind the simpler alkylamino derivatives.

As the aromatic bases are only very sparingly soluble in water, they were dissolved in weak alcohol for purposes of injection. Control experiments without the bases were made with satisfactory results. The α-naphthylamino-acetylcatechol has not been previously described and was prepared by acting upon chloracetylcatechol (1 mol.) with α-naphthylamine (2 mols.) and a little alcohol. It is a faintly yellowish-green coloured crystalline substance, sparingly soluble in dilute spirit and is a very feeble base.

A base ($C_6H_3(OH)_2.CO.CH_2NH.CH_2.C_6H_5$), which may be regarded as intermediate between the two chemical types already described, was obtained by the action of benzylamine upon chloracetylcatechol. The substance is crystalline and readily soluble in alcohol. It had very little effect on the blood-pressure in rabbits, even when injected in fairly large quantities.

Another kind of base was prepared by acting upon chloracetylcatechol with tertiary bases. For example, aqueous tri-methylamine (1 mol.) was digested with chloracetycatechol for some hours, and after adding a drop or two of dilute hydrochloric acid the solution was concentrated and crystallised. Purification is readily carried out by solution in alcohol and precipitation with ether. The substance has the formula $C_6H_3(OH)_2.C = O.CH_2N(CH_3)_3Cl$.

It was found to be *more* active than the corresponding mono-methylamine derivative from which the "adrenalin-like" substance was obtained. In the case of rabbits, so small a quantity as 0·00002 gramme may produce a marked rise. In the comparatively few experiments which were made, it appeared that the substance was very rapidly destroyed after injection, as when quite large quantities were employed the effect was scarcely more prolonged than when the minimal amount necessary to produce maximal stimulation was used.

The reduction of the tri-methylamine derivative did not give products of

much increased activity, thus differing in this respect very distinctly from the mono-alkylamine derivatives.

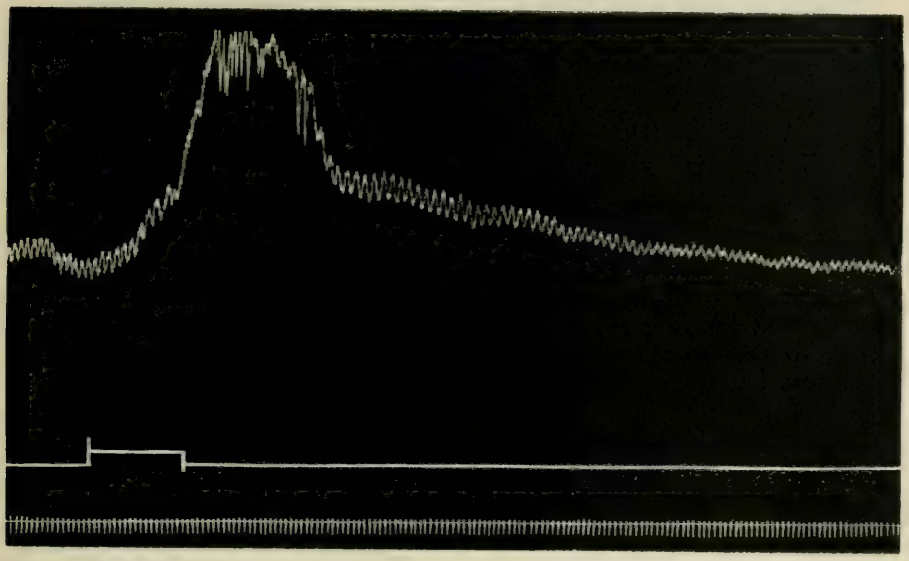
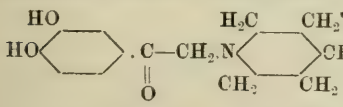


FIG. 3.—Rabbit, 2·5 kilogrammes. Carotid B. P. Vagi divided. Urethane. 0·0005 gramme $C_6H_3(OH)_2.CO.CH_2N(CH_3)_3Cl$. Zero pressure 40 mm. below signal line Time = seconds.

A corresponding base in the aromatic series was prepared from dimethylaniline and chloracetylcatechol, but, like the other aromatic bases, was not effective in producing rise in blood-pressure.

It is well known that injection of piperidine is followed by increase in blood-pressure (5), and it therefore seemed conceivable that if instead of the ordinary amines used in preparing the bases already described, piperidine was substituted, one might obtain products with increased activity. Experiments, however, showed that this was not the case. Piperidino-acetylcatechol,



, was found to be decidedly less active than the corresponding methylamine derivative, and its activity was scarcely increased upon reduction.

It is obvious that the experiments recorded, although ranging over a number of different types of substances, are very incomplete, yet they seem to warrant certain conclusions, and to indicate the lines upon which one must proceed if one wishes to synthesise substances whose physiological properties more or less closely approximate to those of natural adrenalin.

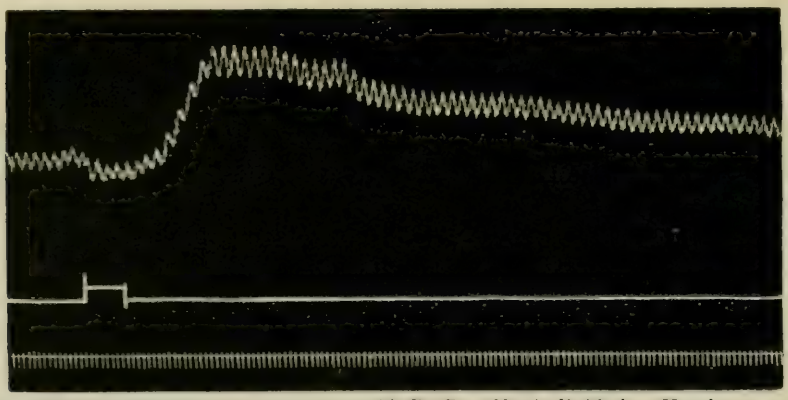


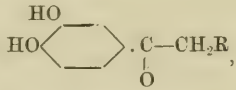
FIG. 4.—Rabbit, 2·5 kilogrammes. Carotid B. P. Vagi divided. Urethane. 0·005 gramme piperidino-acetylcatechol as hydrochloride. Zero pressure 30 mm. below signal line. Time = seconds.

The following deductions are made provisionally, until further experimental evidence is available:—

(i) It appears that the catechol nucleus is essential for the production of physiologically active substances of the type of adrenalin.

(ii) It is of importance that the hydrogen atoms of both hydroxyl groups in the catechol nucleus be unsubstituted.

(iii) An alkyl group of low molecular weight (*e.g.*, methyl, ethyl) attached to the nitrogen tends to produce a much more active substance than when an aromatic group is attached, whilst derivatives of piperidine, heptylamine, and benzylamine occupy an intermediate position.

(iv) The reduction of ketonic bases of the type  , where R is a simple aliphatic group, results in the production of bases with enormously increased physiological activity.

(v) In the substances examined there appears to be a connection between chemical instability and physiological activity, and *vice versâ*.

In conclusion I wish to acknowledge my indebtedness to the Research Fund Committee of the Chemical Society for a grant which has partly defrayed the expenses of the work described in this and the preceding paper.

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(2) Mühlmann, 'Deutsch. med. Wochenschrift,' No. 26, 1896.
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The Synthesis of a Substance allied to Adrenalin.

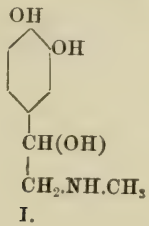
By H. D. DAKIN.

(Communicated by Professor E. H. Starling, F.R.S. Received May 29,—Read June 8, 1905.)

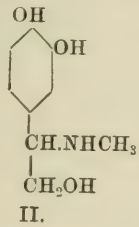
(From the Laboratory of Pathological Chemistry, Lister Institute of Preventive Medicine.)

The isolation of adrenalin from the suprarenal gland by Takamine (1) and by Aldrich (2) in 1901 has led to a considerable amount of work on the chemical nature of this remarkable substance. It is, however, only within the last two years that satisfactory elementary analyses and molecular weight determinations have been made, and the exact structural formula is still unsettled.

Two formulæ have been proposed, either of which will account for most of the observed facts concerning the chemical behaviour of adrenalin, although there are other possible structures which may demand consideration.*



I.

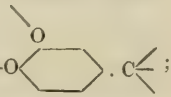
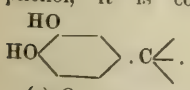


II.

A preliminary note has been published by Friedmann (5), which contains

* It is not proposed to give a *résumé* of previous work, but the following indisputable facts upon which speculations as to structure are based may be briefly stated—there is, however, much evidence of a less direct nature which will be found in the papers by Abel (3), von Fürth (4), Friedmann (5), Jowett (6), Pauly (7), and Stolz (8).

(*a*) Adrenalin, on fusion with alkali, gives catechol, so that an ortho-substituted benzene nucleus is present in the molecule.

(*b*) Adrenalin, on methylation and subsequent oxidation, gives veratric acid—3·4 dimethoxybenzoic acid—pointing to the existence of the grouping  ; and since further adrenalin gives the colour reactions ($FeCl_3$, etc.) of an ortho-dihydric phenol, it is concluded that two hydroxyl groups are present in the ring  .

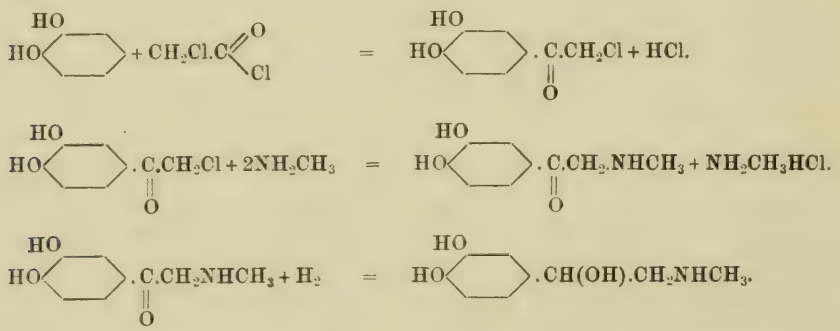
(*c*) On warming with aqueous alkali, methylamine is formed, so that the grouping, $—NH.CH_3$ is present.

(*d*) Adrenalin is optically active, and therefore contains an asymmetric carbon atom which will be situated in the side-chain.

results which would appear to exclude the second formula and support the first. Although criticism must be postponed until further particulars are available, still it may be noted that as at least one of the reactions—the formation of "peradrenalon"—appears to be abnormal, additional experimental evidence is very desirable.*

It is clear that the subject had reached a stage at which synthetical methods might be employed with a possible chance of success, and in January, 1904, I commenced experiments in this direction. A considerable part of my work has, however, been anticipated by workers in the laboratory of Meister Lucius and Brüning (9), and whilst I wish to disclaim any pretensions to priority, I take the opportunity of stating that my results were entirely independently arrived at, and that, owing to the method of publication adopted (Patent Specifications), it is only recently that I have become acquainted with the main portions of their work.

Dziergowski (10) has shown that when catechol is heated with chloracetyl-chloride, or with a mixture of chloracetic acid and phosphorus oxychloride, chloracetylcatechol is formed. It appeared probable that, by acting upon this substance with methylamine and subsequent reduction of the product, a substance would be produced which should have the formula (I) ascribed to adrenalin:—

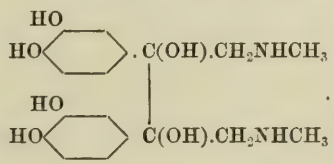


This series of reactions has been carried out, and a substance obtained which has very many of the physiological and chemical properties which are

* It may not be out of place to note that the statement made in abstracts of Friedmann's paper in the 'Biochemisches Centralblatt' and elsewhere to the effect that adrenalin on oxidation gives adrenalon (methylamino-acetyl-catechol) appear to be incorrect. This would obviously be of the utmost importance if true, but Friedmann apparently oxidised the benzenesulphonic acid derivative of adrenalin, and obtained a substance indistinguishable from the benzenesulphonic acid derivative of adrenalon, which is quite a different matter. As the substances are very badly characterised, too much weight must not be placed on the result, which might possibly be explained in other ways.

characteristic of adrenalin. It is true, further, that since the synthetic product would necessarily be optically inactive, one would expect slight differences between the artificial and natural bases, but on the other hand there are certain chemical differences, which will be mentioned later, which I am inclined to think are greater than would be the case if the two substances were stereo-isomeric. This involves the conclusion that either adrenalin or the synthetical base has *not* the structure represented by the formula $C_6H_3(OH)_2.CH(OH).CH_2NHCH_3$.

A substance which I believe to be identical with the base that I have prepared has been obtained by Meister Lucius and Brüning's chemists by the action of aluminium shavings and mercuric sulphate upon methylamino-acetylcatechol. They definitely ascribe the above formula to the substance, and this, if correct, would necessitate an alteration in the provisional structural formula of natural adrenalin. But it must be remembered that the synthetical base has been obtained by a reaction which is well known to give more than one product. Ketones commonly give a mixture of the corresponding secondary alcohol and pinacone upon reduction, either of which substances may constitute the main product. In the reaction under consideration the pinacone would have the following structure:—



Moreover, it must be remembered that the reaction is carried out, as will be seen later, under conditions which may quite well induce further changes in such unstable substances. Owing to experimental difficulties it is impossible at present to settle the point definitely, but I am of opinion that with the evidence at present available one is not justified in selecting preferentially any one of the several possible formalæ for the synthetical base.

The synthetical substance is extraordinarily active as regards the blood-pressure and certain other physiological effects.

Mr. T. R. Elliott was kind enough to examine some of the substance and informs me that so far as the experiments went they revealed no difference between the stimulating action—both motor and inhibitor—of the laboratory and of the animal products. Such close physiological resemblance may be taken as some evidence in favour of a close structural relationship between the two substances.

Experimental.

Chloracetylcatechol is prepared according to Dziergowski's (10) directions by heating catechol (1 mol.) and chloracetic acid (1 mol.) with phosphorus oxychloride equal in weight to the chloracetic acid used. As the reaction in my hands has always followed a course which differs slightly from that described by Dziergowski, the following particulars are given. The mixture should be placed in a large dry flask—at least 1000 c.c. capacity for 50 grammes catechol—and carefully heated in the water-bath. The mixture rapidly becomes fluid and clear, changing after a time to a deep purple colour. The reaction must be carefully watched, as at a certain point a violent evolution of hydrochloric acid with much frothing is liable to occur, which makes it necessary to cool the flask temporarily. Heating is continued until the evolution of hydrochloric acid slackens, and the thick dark-coloured semi-solid mass is dissolved in about three volumes of hot water. The solution is filtered to remove a trace of oily impurity and then allowed to cool. The chloracetylcatechol crystallises out in very well defined crystals, which are usually somewhat discoloured but are easily purified by a single re-crystallisation from boiling water. The yield is about equal in weight to the catechol taken. The dry substance must be handled with care, as although it is without smell, even minute traces of its dust have a very powerful irritant effect on the eyes and nose.

Methylamino-acetylcatechol.—The first product of the action of methylamine upon chloracetylcatechol is a yellow crystalline methylamine salt ($CH_3.NH_3.O.C_6H_3(OH).CO.CH_2Cl$) (10), but this substance on prolonged digestion with excess of methylamine gives methylamino-acetylcatechol, as has also been shown by Stolz (8). For example, 10 grammes of chloracetylcatechol is placed in a stoppered bottle and 25 c.c. of aqueous 33 per cent. methylamine added in portions of 5 c.c. each. The mixture is cooled between the successive additions. The ketone is almost immediately changed into the yellow crystalline salt, and after thorough shaking and allowing to stand for one or preferably two days, the mixture becomes dark brown from the separation of the crude base. The solid is filtered off and washed well with water, alcohol and ether, then shaken with a slight excess of dilute hydrochloric acid. The solution is filtered from any trace of unchanged chloroketone, concentrated and crystallised. Crystallisation is also economically effected by adding alcohol and subsequently ether to the concentrated aqueous solution, the hydrochloride being precipitated in the form of colourless crystals. The free base may be readily obtained from the hydrochloride by precipitating with ammonia and is, when pure, a colourless crystalline

substance, melting at 232° C. and is practically insoluble in water and neutral organic solvents. Most of the salts are readily soluble in water and give a beautifully crystalline compound when shaken with a strong solution of sodium bisulphite. The acetate forms a finely crystalline hydrazone on treatment with phenylhydrazine and a large excess of dilute acetic acid.

Reduction of Methylamino-acetylcatechol.—The reduction offered peculiar difficulties, since the majority of methods applicable for the reduction of ketones were inadmissible owing to the instability of these catechol derivatives in alkaline solution. However, it was found that reduction could be readily carried out electrolytically in acid solution using lead electrodes. The apparatus employed was similar to that devised by Tafel (11), and as it was important to keep the temperature of the liquid in the cathode compartment from rising above 16°, the cell was placed in a freezing mixture.

One part of the base to be reduced is dissolved in the minimum quantity of dilute hydrochloric acid, or preferably the crystalline hydrochloride is taken, and the solution diluted with 15 parts of water. Fifteen parts of 10-per-cent. sulphuric acid are then added to the solution, which is then placed in the cathode compartment. Owing to the sparing solubility of the sulphate of the base, it is advisable to add the sulphuric acid only just before commencing the reduction. The anode compartment is filled with 10-per-cent. sulphuric acid. The reduction is not very rapid, as the time required for reducing 1 gramme of base in a small apparatus, using a current of 3 to 4 ampères, is about half an hour. After the reaction is completed the bulk of the sulphuric acid is removed by adding baryta, and the filtrate contains the sulphate of the new base. A large number of modifications of the above reaction have been tried, but as the products appear to be identical and the above process is the simplest, it is not proposed to give further details, but it may be mentioned that the reduction of the bi-sulphite compound of the base is particularly promising. The aqueous solutions of the salts of the new base, however obtained, may be concentrated *in vacuo*, but are rapidly decomposed if heated on the water-bath.

On addition of ammonia, the free base is thrown out of solution as an amorphous greyish-white precipitate which very readily re-dissolves in acids. The base may be kept suspended in water for some little time without much change, but is so extraordinarily unstable in the dry condition that it has not yet been possible to obtain it in a fit condition for analysis. If one filters the base off as rapidly as possible and quickly removes adherent water by washing with dry alcohol and ether, it is found that the base has already undergone change, and is no longer completely soluble in dilute aqueous acids. This somewhat mysterious behaviour recalls that observed

by Victor Meyer (12) in the case of ω-aminoacetophenone ($C_6H_5.C:O.CH_2NH_2$), which readily forms stable salts, but the free base, when precipitated with ammonia, rapidly changes into a non-basic substance. It is mainly this peculiar property which leads one to conclude that it is unlikely that the synthetical base is the racemic modification of adrenalin, for the natural substance is quite stable in the dry state.

The salts of the base show the usual colour reactions with $FeCl_3$, etc., which are common to adrenalin and other catechol derivatives. Potassium ferrocyanide produces no precipitate in the cold, but gives a greenish precipitate on boiling. The free base, like adrenalin, is not precipitated on addition of sodium acetate to solutions of the salts, although under similar conditions the ketone base, methylamino-acetylcatechol, is precipitated. Picric acid produces no precipitate, but silver nitrate is, as one would expect, rapidly reduced.

If a solution of the sulphate be treated with the exact quantity of barium chloride necessary to remove the sulphuric acid and the solution be concentrated *in vacuo* at about 20° to 25°, a syrupy solution of the hydrochloride is obtained, but which shows no inclination to crystallise. The hydrochloride may be partly precipitated as an extremely deliquescent syrupy mass by adding anhydrous ether to a strong alcoholic solution.

The acetate is obtained in similar fashion from the sulphate by means of barium acetate. It has not been obtained crystalline, and seems to be more unstable than the other salts. On adding an alcoholic solution of oxalic acid to the acetate and then precipitating with excess of anhydrous ether, the oxalate is thrown out as a colourless hygroscopic oil which quickly shows distinct signs of crystallising, but it has not been possible to obtain satisfactory analyses for the salt.

A great many attempts were made to prepare stable crystalline derivatives suitable for thorough investigation, but as this end has not been obtained, it is not proposed to give any account of the products obtained by the action of substances such as benzoyl chloride, benzenesulphonic chloride, acetic anhydride, etc., as none of these derivatives were well characterised substances giving satisfactory analytical results.

Effect of Intravenous Injection of the Reduced Base.—The injection of extremely small quantities of the synthetical substance was found to produce a great rise in arterial pressure. A definite rise may be obtained when one millionth of a gramme of the base in the form of hydrochloride is injected into a rabbit with vagi divided. This and other physiological similarities noted by Mr. Elliott would seem to indicate that the difference between the natural and synthetical bases is but slight, and it is perhaps not inconceivable

that it is to some decomposition product which may be common to both substances that the stimulating property of the bases is due.

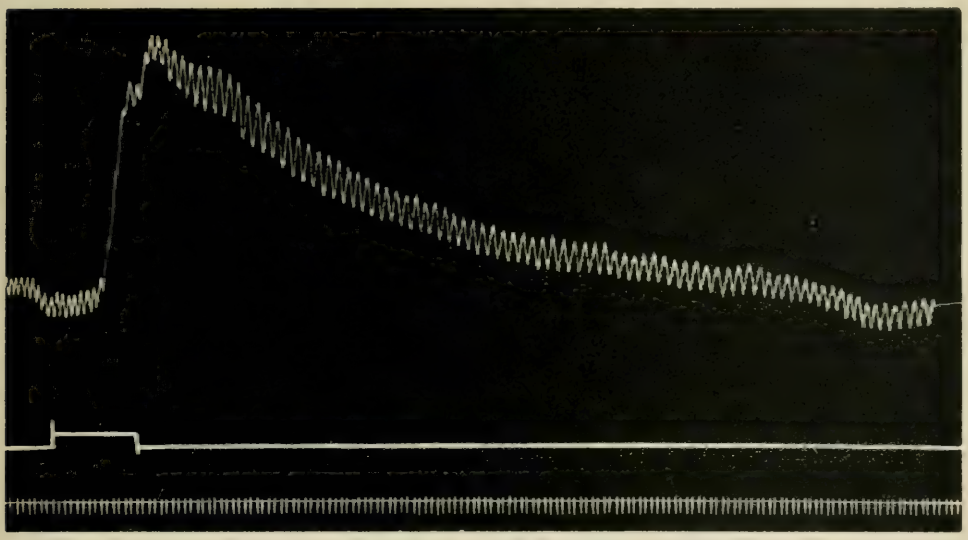


Fig. 1.—Rabbit, 3 kilos. Carotid B. P. Vagi divided. Urethane. Zero pressure 30 mm. below signal line. 0·00002 gramme base as hydrochloride. Time = seconds.

In conclusion I wish to acknowledge my indebtedness to Dr. Leathes for the very large amount of help and advice he has given me throughout the course of the work.

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An Experimental Enquiry into the Nature of the Substance in Serum which influences Phagocytosis.

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(Communicated by Professor J. Rose Bradford, F.R.S. Received July 8, 1905.)

Metchnikoff, and his school, in the face of much opposition, lasting many years, have offered convincing proofs of the importance of phagocytosis in the protection of the animal body against bacterial invasion. The main theses of the Metchnikovian theory are now almost universally accepted, but the exact mechanism of the processes involved is even now the subject of keen controversy. If a highly virulent organism is injected into a susceptible animal, the leucocytes appear to be repelled, and to be unable to deal with the microbe, which multiplies and causes the death of the animal. If, however, the suitable immune serum is injected into the animal before inoculation, the phagocytes attack and devour the invading micro-organisms.

Much discussion has centred round the interpretation of such experiments. The early work of Nuttall and others on the bactericidal action of normal serum, and Pfeiffer's demonstration of the bacteriolysis of cholera and typhoid bacilli by immune sera in the absence of cells, formed the chief basis on which rested the humoral theory, which attributed the protection in such cases to the destructive action of the serum on the microbes. Flügge graphically illustrated the view of the humoralists by likening the phagocytes to the trenches made ready behind the fighting line to receive the conquered dead.

It was found, however, that cases of protection resulting from the use of immune serum occurred where no such bacteriolytic action could be demonstrated; the plague bacillus and the streptococcus may be mentioned as examples. Admitting that the phagocyte plays a part in the protection against these infections, the question must still be considered whether the immune serum has acted on the injected microbes or on the phagocytes. Metchnikoff maintains that the serum stimulates the leucocyte to its activity, whereas many workers, who are quite prepared to admit the important part which the phagocyte plays in the process, hold that the immune serum acts chiefly on the micro-organism.

Metchnikoff's view, however, is not opposed to the idea of the immune substance, or "substance sensibilisatrice," becoming fixed on to the cocci. He admits that this occurs, and that the micro-organisms thus sensitised, though they maintain their vitality and virulence, become more readily the prey of the leucocyte, whose activity is increased by the stimulating action of the "substance sensibilisatrice." In the animal body, under normal conditions, bacteriolysis of the microbe occurs within the phagocyte (Bordet and Levaditi). In experiments *in vitro*, or in the animal body where phagolysis has occurred, free cytase or complement being present, bacteriolysis may occur both outside and inside the phagocyte.

It may be of use here to make a brief reference to a few of the investigations carried out by the followers of Metchnikoff with reference to the influence exerted by the serum, on the one hand on the phagocytes, on the other hand on the microbes. The papers selected to illustrate the subject are by Bordet, Savtschenko, and Levaditi, and their views are referred to only in so far as they touch on these points.

Bordet (1895 and 1897) holds that the specific serum contains a thermostable substance, "sensibilisatrice," which acts on the micro-organisms and prepares them for the thermolabile alexin, or proteolytic ferment, which acts as the solvent. He compares the action of the immune serum to that of a mordant. In certain cases, however, such as in streptococcus infection, the bactericidal action is slight, and in such cases he attributes to the immune serum a stimulating action on the leucocytes. The leucocytes and other cells can perceive the presence of a preventive serum, and under its stimulus they are capable of reacting by movement. They manifest towards the immune serum a pronounced positive chemiotaxis. The activity of the leucocytes in the presence of such serum can be observed *in vitro*.

The bactericidal substance is not uniformly distributed through the plasma, but during life is confined within the leucocytes.

Savtschenko and Melkich (1901), from their study of the processes observed

in recurrent fever, come to the conclusion that the immune substance, or "fixateur," acts as an intermediary body between the micro-organism and the leucocyte, transforming the negative chemiotaxis of the latter into a positive chemiotaxis. They state that the "fixateur" may act in two ways:—

(1) The leucocytes may absorb the "fixateur," and acquire the chemical affinity necessary to enable phagocytosis to occur.

(2) The "fixateur," which is present in a free state in the plasma, becomes fixed on to the spirilla, to which it communicates the chemical affinity for the protoplasm of the leucocytes, and phagocytosis results.

The latter hypothesis is not invalidated by the fact that no Pfeiffer's phenomenon (of bacteriolysis) can be obtained by supplying alexin to the spirilla, since a much smaller quantity of "fixateur" may be necessary for phagocytosis than for bacteriolysis.

Savtschenko (1902), in a later work, comes to similar conclusions, based chiefly on experiments on the phagocytosis of red blood-corpuscles. The immune substance, or "fixateur," can fix itself on the microbe, or on the leucocyte, and has an affinity for the cytase contained in the leucocyte. It is probable, he thinks, that it acts as a stimuline for the phagocyte. He holds that it acts as an intermediary body between the leucocyte and the microbe, and merits fully Ehrlich's designation of "Zwischenkörper" (intermediary body).

Levaditi (1901) showed, by experiments *in vitro*, that cholera vibrios were ingested by the polynuclear leucocytes of the peritoneal exudate of a normal guinea-pig, and that the intracellular vibrios were converted into granules (intracellular, Pfeiffer's phenomenon), whereas the extracellular organisms remained unaltered. From a series of experiments, he came to the conclusion that this result was due to the presence of "substance sensibilisatrice," in sufficient quantity to enable phagocytosis to occur, the complement for the intracellular change of the vibrios into a globular form being, he believed, supplied from the leucocyte itself.

On the other hand, the extracellular solution of the microbes did not take place owing to a lack of complement. The leucocytic origin of the complement will not be approached in the present paper.

Levaditi also showed that the vibrios on to which the "substance sensibilisatrice" had been fixed when introduced into the circulation of a normal animal were rapidly phagocyted, just as they are in the case of an actively immunised animal. The extraphagocytic conversion of the vibrios into the granular form does not take place if sufficient precautions are observed to avoid injury to the leucocytes.

Denys and Leclef (1895) and Denys (1897) showed that the serum of rabbits immunised against streptococcus had a bactericidal action on the streptococcus, but that the serum of the horse had no such action, though it possessed protective properties. The immune substance in the one case acts as intermediary body between the cocci and the alexin; in the other case between the cocci and the leucocytes.

Denys (1897) made comparative tests of the phagocytic action of different sera *in vitro*. Measured quantities of streptococci were introduced into tubes containing leucocytes, and to certain of these were added immune serum, to others normal serum. By plating loopfuls taken from these tubes, and counting the colonies at various periods, he was able to demonstrate a marked diminution in the tubes containing the immune serum, whereas in the tubes containing the normal serum, an increase was observed.

He found that the leucocyte of the immunised animal was no more active as a phagocyte than the leucocyte of the normal animal. The difference in the two cases was entirely due to a property of the serum.

The conclusion arrived at was that the immunity of the rabbit against the streptococcus was due to a modification of the serum, which rendered phagocytosis possible. The immunity in this case is a humoral property acting by the intervention of the phagocytes.

Mennes (1897), using the same method of experimentation with the pneumococcus, obtained similar results to those obtained with the streptococcus by Denys, and concluded that the immunity in this case was due to a modification, not of the leucocyte, but of the serum. The serum had not acquired any bacteriolytic property, but had itself undergone a change, which resulted in the micro-organisms being taken up and destroyed by the leucocytes.

The results of Denys and also of Mennes are not altogether above the criticism made by Metchnikoff, viz., that the occurrence of a certain amount of agglutination would appear to give a diminution in the number of colonies.

Douglas and Wright (1903), adopting and modifying the method suggested by Leishman, have arrived at a very beautiful technique for the study of phagocytosis, and they have published a series of papers on the subject. The details of the method are too elaborate for reproduction here, but the essential point consists in enumerating the bacteria ingested in a number of polymorphonuclear leucocytes, and, by division, obtaining an average, which is taken as the measure of the phagocytic power of the blood. They find that there is present in the normal blood serum a substance which prepares the bacilli, so that they are capable of ingestion by the phagocytes. They call

this effect an "opsonic" effect ("Opsono," "I cater for, I prepare victuals for"), and they use the term "opsonin" to designate the element in the blood-fluids which produces the effect. They find that the "opsonin" is a thermolabile body, *i.e.*, is destroyed by heating to 60° C. for 10 minutes. The leucocyte is an indifferent factor in the matter.

As a result of inoculation with bacterial vaccines, the amount of the opsonin present in the blood may be increased.

Bulloch and Atkin (1905) have confirmed and extended these results of Wright and Douglas. They find that the "opsonin" disappears from serum when the latter is mixed with bacteria at 37° C. or 0° C. The action of heat is to destroy the "opsonin," and not merely to convert it into a "non-opsonisable" modification. It is a simple body, and is not identical with any of the antibodies hitherto discovered in the serum. Leishman, at a discussion on the subject at the Pathological Society, London, supported the "stimuline" view of the action of serum.

Neufeld and Rimpau (1904) find that the immune sera, in the case of the streptococcus and pneumococcus do not stimulate the leucocyte in Metchnikoff's sense, but act on and change the micro-organisms, so that they are secondarily taken up by the phagocytes. They find that the substance which produces this effect is thermostable. A polemic has been waged with Wright as to the identity of this body with the "opsonin" (*cf.* Savtschenko and Markl).*

Hektoen and Ruediger (1905) employed the method of Wright and Douglas, and confirm most of their results. They conclude that the "opsonin" has a complex constitution, there being present a haptophorous group, which fixes on the microbe, and an opsinophorous group, which produces a physical or chemical change in the microbe.

Introduction to Experiments.

The present investigation was undertaken with the view of studying certain questions as to the relation between the phagocytic immunity which occurs in normal animals and in those which have been actively immunised. The serum of a number of animals, chiefly horses, which have been immunised against various microbes, was examined and compared with the serum of normal animals of the same species. At first the method employed was Wright and Douglas', to which reference has already been made.

To save repetition, it may be stated that in almost every case equal volumes of washed leucocytes, of the serum and of the bacterial emulsion

* The writer had observed the thermostability of the substance in Staphylococcus immune serum and in normal serum before hearing of the work of Neufeld and Rimpau. Where the term "the substance" is employed in this paper, it is used as an abbreviation for "the substance which prepares the micro-organisms for phagocytosis."

were employed. The number of ingested cocci were enumerated in 22 polymorphonuclear leucocytes, and the average taken as the phagocytic index.

Estimations were made of the phagocytosis in the case of animals immunised against *Staphylococcus pyogenes aureus*,* *Streptococcus pyogenes*, *B. typhosus*, and *B. dysenteriæ*.

These experiments led to a closer study of the nature of the substance in serum which assists in the process of phagocytosis ; and to some extent this enquiry resolved itself into a study of the relations which the immune substance, amboceptor, substance sensibilisatrice, or fixateur, bears to the opsonin of Wright and Douglas. For this investigation the *Staphylococcus pyogenes aureus* was chiefly employed owing to the convenience with which emulsions, counts, etc., could be made. Two races of the Staphylococcus were used, one highly virulent, recently isolated from the human body, the other an old laboratory culture. No marked differences were observed in the counts obtained from these.

Where the method of Wright and Douglas has been rigidly employed, the results I have obtained have been almost entirely in agreement with theirs. I should not feel disposed, however, to place quite the same reliance as they do on the numerical accuracy of the results which can be derived from the method. Where the leucocytes are very full—*i.e.*, where the counts are high —it is impossible to differentiate results by the method of enumeration. In this paper differences obtained by all refinements of enumeration have been neglected. In all cases an excess of cocci was present. The majority of the counts have been made by two or three observers who did not know the objects in view, so that personal bias was eliminated. Without entering into details, it may briefly be stated that, when using their method, my experiments tend to confirm theirs in that there is in normal serum a substance which prepares the microbe for the leucocyte ; that this substance, or "opsonin," as tested by Wright's method, appears to be destroyed at a temperature of 60° C. for 15 minutes, and that the leucocyte is, in a certain sense, an indifferent factor. By the employment of other methods, results in oppositition to Wright and Douglas' have been obtained.

When, however, the question of the thermolability of the "opsonin" was investigated in another manner, the results obtained were opposed to the view that the substance was destroyed at the temperatures named by Wright. Indeed, it was found that the heated immune sera, even on testing by Wright's method, gave high phagocytic indices. These results suggested a re-investigation of the effects of temperature by other methods.

* Referred to hereinafter as "Staphylococcus."

One method employed was that of adding a measured quantity of a microbial emulsion to a certain volume of serum which had been heated to 60° for 20 minutes, allowing this to stand for varying periods at 37° C., then centrifugalising and ascertaining whether the centrifugalised cocci were capable of being taken up by the leucocytes.

Experiment.—0·5 c.c. of an emulsion of Staphylococcus (= ½ agar tube) was added to 5 c.c., of a normal horse serum, which had been heated to 60° C. for 20 minutes. The cocci, separated by centrifugalisation and made into a suitable emulsion with normal salt solution, were compared with normal fresh cocci in Wright's tubes.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Emulsion of fresh cocci	1	4
+ Heated normal horse serum	1	
+ Normal horse leucocytes	1	
Emulsion of prepared cocci	1	60
+ Normal salt solution	1	
+ Normal horse leucocytes	1	

Experiment.—0·1 c.c. of an emulsion of Staphylococcus (= 1/10 of an agar tube) was mixed with 1 c.c. of normal human serum, which had been heated to 60° C. for 20 minutes. This mixture was placed for 15 minutes at 37° C., and then the cocci were centrifugalised from the mixture and made into a suitable emulsion with normal salt solution.

These prepared cocci and fresh cocci were used for a comparative test in capillary tubes in the ordinary manner.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh cocci	1	0
+ Heated normal serum	1	
+ Leucocytes (human)	1	
Prepared cocci	1	60
+ Normal salt solution	1	
+ Leucocytes (human)	1	

A large number of similar experiments which need not be detailed here were done with concordant results; in several cases a smaller proportion of serum to bacterial emulsion was used.

Such results were obtained in the cases of normal human serum (four samples), horse serum, goat serum, rabbit serum, guinea-pig, and rat serum. Normal horse serum which had been kept for four years still retained this property.

These experiments prove that though the results obtained by using Wright's method seem to demonstrate that the substance capable of preparing the

microbes for phagocytosis is destroyed by heating for 20 minutes in 60° C., only a fractional destruction of the substance occurs. The loss in the case of sera, such as normal sera, which contain only a comparatively small quantity of the substance, is so great that a method where very small quantities are used makes the demonstration and estimation of the substance impossible.

On the other hand, where a large amount of the substance is present, as in certain immune sera, the heating to 60° C. for 20 minutes, or for even much longer periods, leaves enough of the substance undestroyed to give results by Wright's method. Indeed, the counts obtained may be higher than with fresh normal serum of the same species.

One or two experiments may be quoted as examples of a large number done which gave consistent results:—

Experiment.—

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh normal rabbit's serum	1	9
+ Suspension of Staphylococcus.....	1	
+ Leucocytes of horse	1	
Heated normal rabbit's serum	1	0·2
+ Suspension of Staphylococcus	1	
+ Leucocytes of horse	1	
Immune rabbit's serum (heated to 60° C. for 20 minutes)	1	20
+ Suspension of Staphylococcus	1	
+ Leucocytes of horse	1	

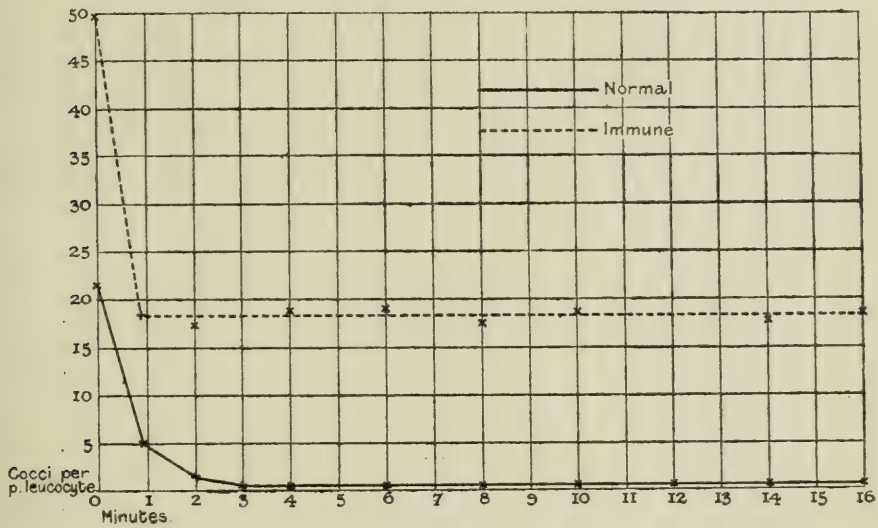
The serum of the normal horse, even when heated to 60° C. for 20 minutes, gave in certain cases, by Wright's method, fairly high counts. It contained a larger quantity of the substance than heated normal rabbit's serum.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh normal horse serum	1	30
+ Suspension of Staphylococcus	1	
+ Leucocytes of horse	1	
Fresh normal horse serum (heated to 60° C. for 20 minutes).....	1	8
+ Suspension of Staphylococcus	1	
+ Leucocytes of horse	1	
Immune heated serum of horse	1	60+
+ Suspension of Staphylococcus	1	
+ Leucocytes of horse	1	

These experiments show that in an immune Staphylococcic serum enough of the substance remains undestroyed by heating to be demonstrable by the use of Wright's method.

When fresh serum, either normal or immune, is heated to 60° C. for various periods, one finds that, as estimated by Wright and Douglas' method, there is a great fall in the first two minutes, and after that the curves run almost parallel to the base line. In the case of normal serum the fall is so great that the curve may reach the base line.

The accompanying chart gives a graphic representation of what happened



on heating a normal rabbit's serum and an immune Staphylococcus rabbit's serum for different periods up to 16 minutes. The number 50 applied to the original strength of the immune serum is only approximate, as in such high counts accuracy is impossible. It will be seen that after one minute's heating the immune serum fell to about 18 cocci per leucocyte, and, allowing for experimental errors (the average of a large number of counts was taken), it then runs parallel to the base line.

The normal serum fell from 20 to 5 at the end of the first minute, to 1 at the end of the second minute, and then ran almost parallel to the base line, the average phagocytic index of a number of counts being about 0·5.

The results after 30 minutes showed that no great change had occurred.

One must remember that Wright and Douglas' method probably demonstrates the presence of the "opsonin" over only a very short range. The brief time during which the substance is allowed to act on the cocci probably admits of only fairly high concentrations being indicated. If one compares

what occurs in the case of agglutination, only the very strongest agglutinating sera would be regarded as giving positive results if 15 minutes were put as the limit of time for the serum to act on the microbes.

As has been shown by the experiments quoted, the fall in the curve does not indicate complete destruction of the substance, as was stated by Bulloch and Atkin, but merely indicates that the substance has reached a concentration below that demonstrable by Wright and Douglas' method.

This view is emphasised by the experiment detailed in the following section.

Effect of Continued Heating on the Substance in Serum.

Two series were prepared with normal horse serum heated at 60° C. for different periods. In each tube 1 c.c. of the heated serum was placed, and to the tubes of the one series 0·08 c.c., to those of the other 0·05 c.c., of a coccal emulsion was added. The mixtures in each case were put for 15 minutes at 37°, then centrifugalised, and the centrifugalised cocci tested in the usual way. In most cases the leucocytes had to be noted simply as full, but where an obvious fall in the number of ingested cocci was observed, an enumeration was made.

Time for which serum was heated at 60° C.	Tubes to which 0·08 c.c. coccal emulsion added. No. of cocci per leucocyte.	Tubes to which 0·05 c.c. coccal emulsion added. No. of cocci per leucocyte.
20 minutes	Full	Full
40 ,,	Full	Full
60 ,,	Full	Full
2 hours	36	Full
3 ,,	30	44
4 ,,	30	21

This experiment proves that normal horse serum after being heated for four hours at 60° C. still contains enough of the substance to prepare a large number of the cocci for phagocytosis. With the quantities of coccal emulsion employed in this experiment a fall was observable with the larger quantity only after two hours' and with the smaller quantity after three hours' heating.

On the Influence of Temperature on the Rate of Combination between the Substance and the Cocci.

0·1 c.c. of an emulsion of Staphylococcus was added to 2 c.c. of normal human serum, which had been heated to 60° C. for 25 minutes. The emulsion was at once divided into two parts, and one part placed at 37° C., and the other at about 6—8° C., in the ice-chest. At the end of half an hour they were centrifugalised, the centrifuge buckets having been cooled down. The centrifugalised cocci in the two cases were then tested.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Coccal emulsion which had been kept at 37° C.	1	22
+ Human leucocytes	1	
Coccal emulsion which had been kept at 6—8° C.	1	2·2
+ Human leucocytes	1	

This experiment seems to show that the substance acts on or becomes attached to the cocci much more slowly at low than at high temperatures.

Microbes which have been placed in Contact with Immune Serum are capable of taking up an Excess of the Substance which can again Diffuse into the Surrounding Fluid.

0·5 c.c. of an emulsion of Staphylococcus was put in contact with 2 c.c. of a heated immune serum and left in contact at 37° C. for 1 hour. The cocci were then separated by centrifugalisation, washed and put into 1 c.c. of an immune serum, X, and left in contact for an hour at 37° C., then centrifugalised very carefully so that all the cocci were thrown down.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh serum "X"	1	37
+ Staphylococcic emulsion	1	
+ Washed horse leucocytes	1	
Serum, X, through which prepared cocci have been centrifugalised	1	48
+ Staphylococcic emulsion	1	
+ Washed horse leucocytes	1	

This points to a diffusion of the substance into the serum from the cocci.

Experiment.—Cocci which had been treated, as in the previous experiment, with immune serum, after being centrifugalised and rapidly washed with normal salt solution, were left in contact for 24 hours with normal salt solution, which was then tested for the presence of "opsonin."

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Washed leucocytes of horse	1	0·5
+ Normal salt solution	1	
Washed leucocytes of horse	1	21
+ Normal salt solution, from above prepared cocci	1	

A number of experiments were carried out with the view of ascertaining the relation of the substance in normal serum to the substance in immune serum.

Experiment to ascertain whether Cocci fully occupied by the Substance from Normal Serum are capable of absorbing the Substance from Immune Serum.

An emulsion of Staphylococcus, prepared in the usual manner, was added to normal horse serum which had been heated for 25 minutes to 60° C. The mixture was placed at 37° C. for 15 minutes, and the cocci were then centrifugalised till all were removed from the fluid. The supernatant fluid was then removed and the cocci rapidly washed with normal salt solution and again thrown down, great care being taken to avoid any loss of the cocci during the process.

To equal parts of a certain Staphylococcus immune serum from the horse, also heated to 60° C. for 20 minutes, there were added equal parts of the sensitised cocci and of fresh normal cocci. These mixtures were then centrifugalised, and the phagocytic indices of the supernatant fluids estimated and compared, the usual proportions of washed leucocytes and Staphylococcus emulsion being used.

	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Original heated immune serum	24
Original heated immune serum through which prepared cocci had been centrifugalised	13
Original heated immune serum through which normal cocci had been centrifugalised	4

These numbers are the mean of three counts each of 22 polymorphonuclear leucocytes.

A similar experiment carried out with a dilution of one in five of a similar serum of greater potency gave the following numbers :—

	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Phagocytic index of the dilution of original Staphylococcic immune serum of horse	30
Ditto through which prepared cocci had been passed	24·3
" " normal " "	8

These two experiments seem to show that the cocci occupied by the substance from normal serum are incapable of taking up the substance from immune serum, whereas fresh cocci are capable of removing a large proportion of the substance.

The converse experiment was carried out. In this case the cocci were first prepared by contact with immune serum and then passed through a normal serum. For convenience of estimation by Wright's method, instead of a normal heated serum a normal fresh unheated serum was employed.

Equal measured quantities of fresh cocci and of cocci prepared as in the previous experiments by contact with heated immune serum of the horse were added to equal

volumes of normal horse serum, centrifugalised, and the supernatant fluid, which was then free from all organisms, tested for phagocytic power, normal washed horse leucocytes being used,

	Average number of cocci per leucocyte in 22 polymorphonuclear leucocytes.
Original serum	40
Serum through which prepared cocci had been passed	30
Serum through which fresh normal cocci had been passed	2·3

The repetition of this experiment with freshly prepared material gave the following numbers :—

	Average number of cocci per leucocyte in 22 polymorphonuclear leucocytes.
Freshly prepared serum	35+
Ditto through which the prepared cocci had been passed	27
Ditto through which fresh normal cocci had been passed	2

In carrying out these experiments care must be taken that excess of substance is not left adhering to the cocci, in which case it may diffuse out into the surrounding fluid. See previous experiments. To obviate this happening the prepared cocci must be rapidly washed in normal salt solution.

These experiments seem to show that the cocci occupied by the substance from immune serum are incapable of taking up much of the substance from normal serum, whereas fresh cocci are capable of removing a large proportion of the substance.

Tests of the Serum of Animals Immunised against the Streptococcus, Typhoid and Dysentery Bacilli.

Streptococcus.

The serum of three horses immunised against many races of streptococci was examined with the following result :—

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Horse 1—		
Washed leucocytes of horse	1	
+Serum of immune horse	1	9
+Emulsion of streptococcus from agar	1	
Washed leucocytes of horse	1	
+Serum of normal horse	1	4
+Emulsion of streptococcus from agar	1	
Horse 2—		
Washed leucocytes of horse serum	1	
+Serum of immune horse	1	12
+Emulsion of streptococcus from agar	1	

Horse 2—*continued.*	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Washed leucocytes of horse serum	1	3
+ Serum of normal horse	1	
+ Emulsion of streptococcus from agar	1	
Horse 3—		
Washed leucocytes of horse serum	1	23
+ Serum of Horse 3	1	
+ Emulsion of streptococcus from agar	1	
Washed leucocytes of horse serum	1	4
+ Serum of normal horse	1	
+ Emulsion of streptococcus from agar	1	

Typhoid Bacillus.

	Vol.	Bacilli contained in 22 polymorphonuclear leucocytes. No. of ingested bacilli per leucocyte.
Serum of normal horse heated to 60° C. for 20 minutes	1	22
+ Washed horse leucocytes	1	
+ Emulsion of typhoid bacillus	1	
Serum of immune horse heated to 60° C. for 20 minutes	1	49
+ Washed horse leucocytes	1	
+ Emulsion of typhoid bacillus	1	

Dysentery Bacillus (Shiga).

Serum of normal horse heated to 60° C. for 20 minutes	1	1·5
+ Washed horse leucocytes	1	
+ Emulsion of dysentery bacillus	1	
Serum of immune horse heated to 60° C. for 20 minutes	1	25
+ Washed horse leucocytes	1	
+ Emulsion of dysentery bacillus	1	

In the case of the typhoid and dysentery bacilli considerable difficulty was found in estimating the phagocytosis on account of the agglutination. The leucocytes, crammed full of bacilli, were found in some cases lying in groups close to bacillary clumps, in other cases it seemed as if the leucocytes when full of microbes tended themselves to become agglutinated.

It is possible that an agglutination of the bacilli towards the leucocytes may be a part of the process which enables the leucocyte by the movements of its own protoplasm to englobe the microbe. The extraordinary rapidity

with which a bacterial field is cleared of micro-organisms suggests such an occurrence.

An interesting observation was made in this connection, viz., that organisms after being stained with fuchsin, which has a strong agglutinating action, were capable of ingestion, whereas the same organisms killed by heat were refused by the leucocyte.

It was observed that the normal serum of the same horse heated to 60° C. for 20 minutes had a considerable "opsonising" action on the typhoid bacillus, whereas it had little effect on the dysentery bacillus. Twenty-two typhoid bacilli were ingested per leucocyte compared with 1·5 dysentery bacilli.

Summary and Remarks.

An immune staphylococcic serum obtained from the rabbit when heated to 60° C. still contains a substance capable of preparing the cocci for phagocytosis. An identical result was obtained in the case of an immune staphylococcic serum from the horse. Efforts were made to ascertain whether this substance was identical with the substance in normal serum giving rise to the same effect, *i.e.*, to the "opsonin" of Wright. It was found that when the cocci were added to a fairly large volume of normal serum which had been heated to 60° C. for 20 minutes, incubated for 15 minutes and centrifugalised, they were rapidly phagocyted.

It appeared, therefore, that the heating to 60° C. had produced only a fractional destruction of the opsonin, which in the case of normal serum was present in such small amount that it was no longer measurable by Wright and Douglas' method.

This view was confirmed by heating for various periods both normal and immune serum and comparing the fall resulting. The curves obtained in both cases were similar. The serum of various animals, the goat, rabbit, horse, guinea-pig, rat, and human were tested for the thermostability of the substance. In all it was found to be thermostable when tested in the way mentioned, but more of it appeared to be present in the serum of the horse than in the serum of, *e.g.*, man and rat, since in the case of the horse it was found to be present, after heating, in an amount which was still capable of being demonstrated by Wright's method. Horse serum heated for four hours to 60° C. still contained a large amount of the substance.

Experiments were carried out with the view of ascertaining the relation of the substance in normal serum to the substance in immune serum.

Cocci which had been prepared by contact with normal serum so that they were probably fully occupied by the substance were passed through heated

immune serum. On removal of the cocci by the centrifuge the supernatant fluid, when tested with fresh cocci, was found to have lost little, or none, of its original strength; whereas the same fluid through which fresh cocci had been centrifuged had lost practically all its power. The converse experiment gave a quite similar result. In this case, however, care must be taken to avoid the cocci being overloaded with immune serum, in which case they are capable of giving off some of their substance into the suspending fluid.

In this case also it is more convenient to use unheated normal serum, which enables one to easily estimate the opsonic power by Wright and Douglas' method. These two groups of experiments are strongly in favour of the substance in normal serum being identical with the substance in immune serum.

The relation which the "opsonin" of Wright and Douglas bears to the "immune substance," or "fixateur" in so far as the latter influences phagocytosis, as shown by Savtschenko among others, must be briefly discussed.

If the "opsonin" of normal serum were completely destroyed by heating to 60° C. for 15 minutes we should be compelled to assume that it was a separate and new body, and that the increase in the serum of the property of preparing the microbes for phagocytosis, which results from the injection of bacilli, was due to an entirely different body, since the substance resulting from such bacterial injections is markedly thermostable, even when tested by Wright and Douglas' own method.

The experiments which are recorded in this paper, however, show that the destruction by heating of the "opsonin," even of normal serum, is only fractional, and that its apparently complete disappearance is due to the method of observation employed, which demonstrates its presence over a very short range. According to the ordinary use of the word in such investigations the body is thermostable.

The fact that this specific substance is present in small amount in normal serum is in accord with the numerous observations of the occurrence of immune substance in normal sera. One need only refer to the normal antitoxin (*e.g.*, of diphtheria), anti-ferments, etc., and to the fact that the bacteriolytic and hæmolytic actions of normal serum are due to the presence in the serum of an immune substance plus a complement, as has been firmly established by the work of Pfeiffer, Bordet, Moxter, Ehrlich and Morgenroth, and others. In giving the name of "opsonin" to the substance which becomes attached to the micro-organisms and prepares them for phagocytosis, Douglas and Wright have, therefore, named a property of serum which had already been recognised by a number of different workers.

Whether free complement may take part in the preparation of the microbe is difficult to determine. From the experiments detailed in this paper it is

certain that it is not a necessary participant in this action. At the same time it is not improbable that the immune body when aided by complement may act more powerfully, and that the sudden fall in the "opsonic" power of both normal and immune serum on heating is due to the destruction of the complement. I may revert to this subject on another occasion.

Metchnikoff's statement in regard to natural immunity, that the leucocytes undertake the struggle against the microbes and free the organisms from them without the need of previous help on the part of the humors, is apparently largely based on Bordet's and Gengou's results obtained by their method of testing for the presence of "fixateur." The results obtained by the use of that method are not above criticism. That the amount of fixateur present in normal serum compared with immune serum is small is true, but, as suggested by Savtschenko, the amount necessary to prepare the organism for phagocytosis may be small compared with that necessary for bacteriolysis. When microbes, *e.g.*, streptococci, injected into the peritoneal cavity, come in contact with the phagocytes, at first they are englobed by these, but soon some are observed to be free and to multiply rapidly, apparently having the power of repelling leucocytes. Bordet and Savtschenko interpret this to mean that the cocci have acquired during this brief period a new property which gives rise to a negative chemiotaxis of the leucocytes.

In the light of my experiments another view may be taken, viz., that the first organisms injected are phagocyted, because they have been sensitised by the immune substance present in the normal serum. This being small in amount is soon exhausted, and the few organisms which may have escaped its action are able to multiply, and either are indifferent to the leucocytes or exercise their repelling influence on them in the absence of the naturally present immune substance. In such a case the indifference displayed by the phagocytes to the cocci, or it may be the repulsive force of the cocci, is not a newly-acquired property, but is inherent in the cocci, and is only overcome by the presence of the immune substance which acts as intermediary between the micro-organism and the leucocytes.

Conclusions.

1. That, as has been shown by a number of workers, *e.g.*, Denys, Metchnikoff, Savtschenko, Levaditi and others, there is produced in the blood serum of animals actively immunised by bacterial injections a specific immune substance which has among its properties that of preparing the microbe for phagocytosis.

2. That this immune substance is thermostable, resisting a temperature of 60° C. for several hours.

3. That in normal serum there is present a substance having a similar action and which also resists a temperature of 60° C. for hours, and may persist in the serum of the horse for years.

4. That the experiments recorded in this paper tend to confirm the idea that the substances are identical, *i.e.*, that in normal serum there is present a small amount of the immune substance having the property of preparing the microbes for phagocytosis.

5. That cocci fully occupied by the substance from heated immune serum when passed through fresh normal serum do not remove the substance from normal serum, whereas fresh cocci remove a large part of it.

6. That the converse of the above is also true, viz., that cocci fully occupied by the substance from normal serum do not remove the substance from immune serum, whereas fresh cocci do.

7. That the thermostable substance in normal serum is no doubt identical with the "fixateur" or "substance sensibilisatrice" of the French school and with Wright and Douglas' "opsonin."

Seeing that the terms "fixateur" and "substance sensibilisatrice" which have been employed by Metchnikoff's school to include the property of preparing the microbes for phagocytosis are used to designate a number of other properties of immune serum, it may be convenient to adopt Wright and Douglas' term of "opsonin" for the particular property in question. The only danger attached to such a course is that one might be led to regard the "opsonin" as actually a different substance and not merely a property of immune serum.

I wish here to express my thanks to Drs. MacConkey and Petrie for the kind assistance they have given me in making a large number of the enumerations.

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THE ACTION OF ANÆSTHETIC QUANTITIES OF CHLOROFORM UPON THE BLOOD VESSELS OF THE BOWEL AND KIDNEY; WITH AN ACCOUNT OF AN ARTIFICIAL CIRCULATION APPARATUS.

By E. H. EMBLEY, M.D., *Hon. Anæsthetist to the Melbourne Hospital*, and C. J. MARTIN, M.B., D.Sc., F.R.S., *Director, Lister Institute of Preventive Medicine, London.* (Seven Figures in Text.)

In a paper on the causation of death during the administration of chloroform by Embley [1] [2] [3], evidence was adduced to show that although it was quite true, as maintained by Gaskell and Shore [4], that in the early stages of administration the direct effect of chloroform on the vasomotor centre was stimulative, nevertheless the net effect upon the organs experimented with (kidney and bowel) was more or less vaso-dilatation. This diminution of vascular tone was not easily demonstrated by the ordinary method of taking simultaneous oncometric and blood-pressure records, as the marked weakening of cardiac contractions was usually such as to mask any effect due to arterial relaxation.

In a few records, notwithstanding a fall in blood-pressure from lessened cardiac contractions, the volume of the bowel or kidney actually increased (*vide* Figs. 10 and 11, Embley, *op. cit.*).

Embley showed that whether the effect on volume due to vaso-dilatation were, or were not obscured by the fall in pressure from diminished cardiac contractions, the fact that vaso-dilatation had occurred could always be demonstrated on discontinuing the chloroform after a few minutes. As the chloroform is dissipated through the lungs the cardiac contractions improve in force and the pressure rises, and with it the volume of the organ, but the latter rise is out of all proportion to the former and greatly exceeds its previous height (*vide* Figs. 8 and 9, Embley), showing that vaso-dilatation existed and that the heart recovered more quickly than the vessels.

The view was advanced by Embley that the action of chloroform upon the vasomotor mechanism was such that, although it at first stimulated to some extent the medullary centres, its action was from the

beginning paralytic upon subsidiary mechanisms, *e.g.* either in the cord, or the nerve endings in muscle, or the muscle of the arterial wall, or all three.

From the analogy with cardiac muscle, it was suggested as not improbable that the arterial muscle itself might be highly sensitive to the action of chloroform, and that the vaso-dilatation which was shown to occur was occasioned in this way.

Since the publication of Embley's work Schäfer and Scharlieb[5] have published a short account of the action of chloroform upon blood vessels, in which they complain that they cannot find "on what direct evidence the almost universal opinion of physiologists that chloroform produces dilatation of the arteries is based." These observers found that the addition of ·05 to ·5 % by volume of chloroform to Locke's saline fluid, which was being perfused through the body of a frog, invariably diminished the flow; from which they infer that in the frog the action of chloroform is to produce contraction and not dilatation of arterioles.

We have never performed such an experiment, but we have no doubt from the statement of Prof. Schäfer and Dr Scharlieb that the perfusion through the vessels of a frog of saline containing chloroform in this amount does constrict the arteries, as we find that solutions of ·2 to ·5 % do the same in the isolated organs of a warm-blooded animal. We think, however, that the fact that they regard ·05 % by volume as "relatively small amounts" is misleading, for under no circumstances could such amounts gain access to the blood of an intact warm-blooded animal by respiration. Such an amount in a water solution at 37° C. would be in equilibrium with an atmosphere containing 4·2 % of chloroform, and the heart would be completely paralysed long before the tension of chloroform in the blood rose to this value, as was shown by Embley[1] (Fig. 1 and p. 820), by Tunnicliffe and Rosenheim[9], and Sherrington and S. C. M. Sowton[8][1].

In the report of the Chloroform Commission of the British Medical Assoc. Sherrington and Sowton[7] experimenting with the isolated mammalian heart also come to the conclusion that chloroform causes constriction of the arterioles. Their sole reason for this conclusion is the fact that the amount of fluid passing through the coronary circulation considerably diminishes when saline containing chloroform is

[1] That during inhalation the tension of chloroform in blood rises slowly, owing to weak chemical combination with the proteids and hæmoglobin, follows from the experiments of Moore and Roaf. *Proc. Roy. Soc.*, LXXIII. p. 382. 1904.

substituted for the simple saline solution. This is quite true, but we do not think it necessarily bears the interpretation placed upon it.

We have made a large number of experiments upon isolated mammalian hearts for a variety of purposes and always found that, the pressure and other conditions remaining constant, substances (including atropin) which diminished the efficiency of the cardiac contractions, diminished the flow through the heart. Accordingly we cannot accept Sherrington and Sowton's experiments as demonstrating vaso-constriction.

Quite recently Schäfer and Scharlieb [6] have published a further account of their observations on the action of chloroform upon the arteries. In their preliminary survey of the literature they very kindly refer to the work which forms the subject of the present paper, the results of which were communicated to the Physiological Society on February 20, 1904, but not published. In referring to our work Schäfer and Scharlieb have, however, fallen into error, as our observations were not, as they state, confined to oncometric measurements with the kidney.

Schäfer and Scharlieb have extended their observation on the effect of perfusion of Ringer's fluid containing chloroform, to isolated mammalian organs, viz. kidney, heart, and limbs, and have arrived at the result that, whereas with the last two chloroform of various strengths produced either no effect, or else constriction, with the kidney, on the other hand, solutions containing 1 in 20,000 to 1 in 1500 of $CHCl_3$ occasioned dilatation, but stronger solutions produced constriction.

Our own observations have been confined to determining the effect upon the arteries of the isolated bowel and kidney, of chloroform in such quantities as may be present in the blood, when the air respired contains 2 to 3 % of chloroform vapour. We have found that in both cases the dilatation produced is very marked, but more so upon the vessels of the bowel.

The results of Schäfer and Scharlieb and ourselves are not contradictory, but apparently supplementary, and it is interesting to find that the vessels of different parts of the body react in such an opposite manner to the same poison. In chloroform poisoning the dilatation would appear to be more or less confined to the splanchnic area.

On undertaking the present investigation we first of all made a number of experiments with simultaneous records of blood-pressure and volume of organs, similar to those already published by Embley (1902). Fig. 1 records an instance in which notwithstanding the fall of blood-pressure the volume of the bowel increased. About half a minute after

2·2 % chloroform was pumped into the lungs, the arterial pressure commenced to fall, and shortly after, the volume of the organ rose. The chloroform was administered for 2½ minutes. Six minutes after the replacement of the chloroform by ether the volume and blood-pressure had returned to their previous dimensions.

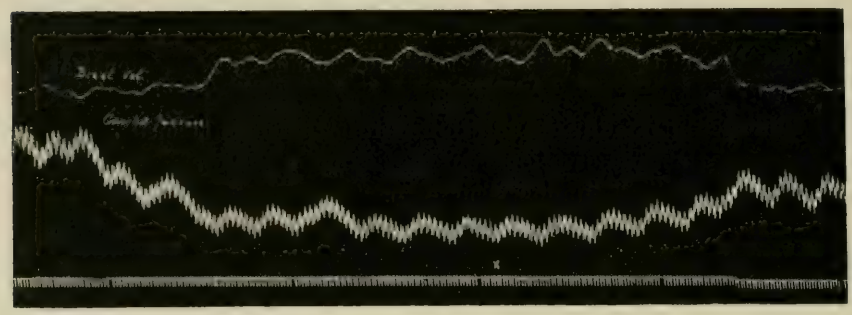

Fig. 1. Dog, Vagi cut, + chloroform given, + + chloroform replaced by ether.

On examining our collection of curves it became obvious that those animals, in which at the time of introducing the chloroform the blood-

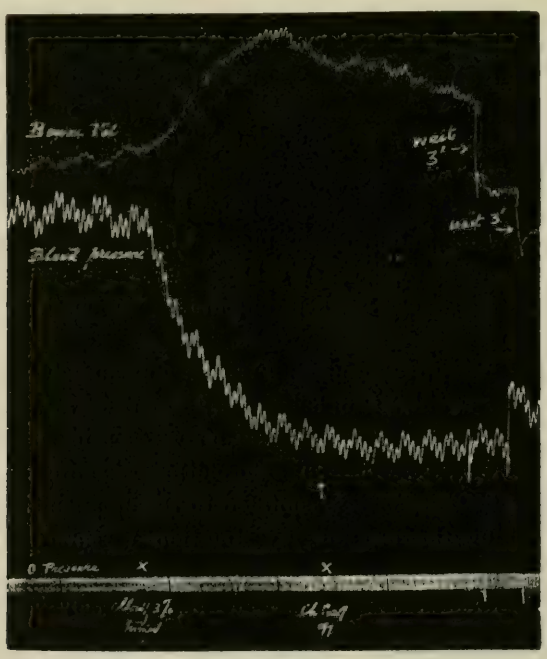

Fig. 2.

pressure was high, and in which there was presumably a considerable measure of vaso-constriction, demonstrated oncometrically this paralysing action of chloroform on the vasomotor apparatus to the best extent.

We therefore artificially increased the existing vaso-constriction by the injection of strychnia, and Fig. 2 shows the effect upon the blood-pressure and bowel volume of 3 % chloroform in the air administered to a curarised dog which had previously received a small dose of strychnia. The chloroform was given during $\frac{3}{4}$ minute. The effect is exaggerated and almost instantaneous. The vertical lines to the right of the tracing indicate periods of 3 minutes during which the drum was stationary.

We have also performed a series of experiments in which an artificial circulation was sent through the isolated bowel or kidney, in order to determine in this way the action of chloroform in anæsthetic quantities upon the peripheral vessels separated from the central nervous system. Only undiluted defibrinated blood was used, and this was perfectly oxygenated by having a double circulation in which it was pumped first through the isolated lungs of the animal, and then by a second pump through the organ (the small intestine or kidney) and returned to the lung circulation.

The chloroform was administered in the air with which the lungs were rhythmically inflated by a mechanical pump. The strength of chloroform in the air used for inflation was controlled and determined by the method used by Embley and described in the *Brit. Med. Journ.*, April, 1902.

A simple form of artificial circulating apparatus which we have found to work well and which permits of every adjustment (Figs. 3 and 4), was employed. It has three important advantages: (1) It is composed entirely of glass and rubber and can be easily cleaned by pumping hot soda water through it.

(2) It requires no very special apparatus and can be constructed in any laboratory.

(3) It, when once going, requires little looking after, so that it may be left for an hour or two, a great advantage in lengthy experiments.

The two pumps *R* and *L* consist of the bulbs of small Higginson seamless "Antrum syringes," capable of containing about 25 c.c. of fluid. The tube is cut off close to the bulbs. Allen and Hanbury's feeding-bottle valves with the nipples slit down with a razor, so as to allow them to open more easily, and fitted into glass tubes, are attached in the position marked *V* (Fig. 3).

The bulbs are compressed by being inserted between two pieces of wood hinged at the back, and the lower ones of these are hollowed out in order to keep the bulbs in position during compression (Fig. 4).

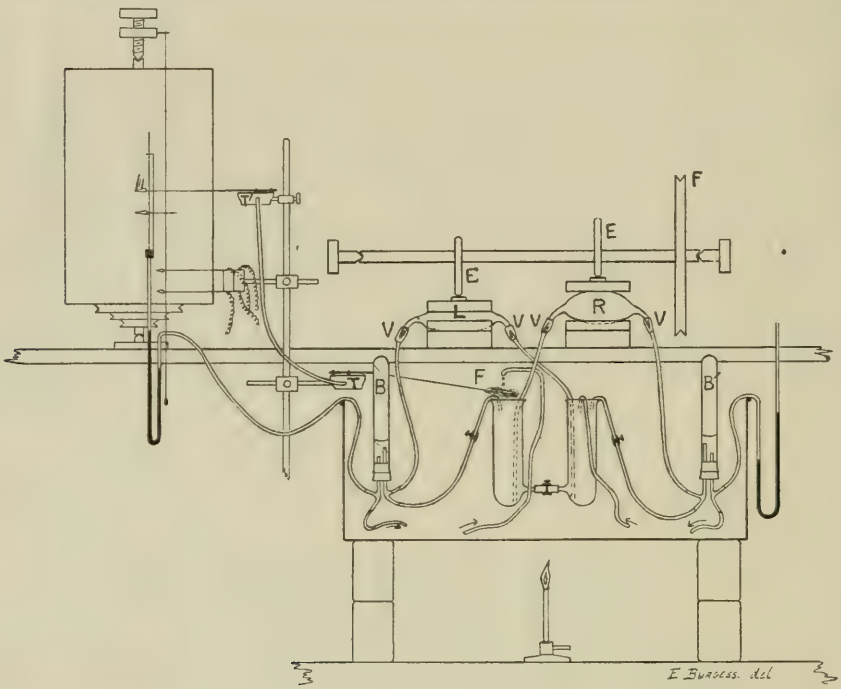

Fig. 3.

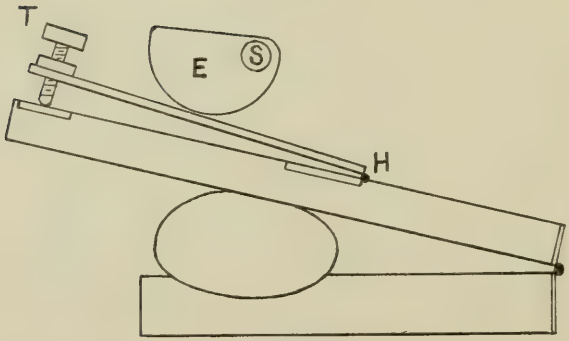

Fig. 4.

They are alternately squeezed and allowed to expand by means of the excentrics E on the shaft S which is driven by the pulley P from some form of motor, the speed of which can be regulated at will. The

"throw" of the excentric and therefore the amount of liquid ejected with each stroke is regulated for both pumps independently by altering the inclination of the piece of stout brass hinged at *H* (Fig. 4) by means of the thumb-screw *T* (Fig. 4).

Each pump draws blood from one of the venous reservoirs *A* and *V*, and from the delivery of each a rubber tube leads off and is connected with a three-way piece of glass tubing. The upper end of the three-way piece is passed through a rubber cork which is inserted into an inverted stout-walled test-tube (*B* and *B'*, Fig. 3) and the side tube is connected to a mercury manometer. A second three-way tube is inserted through the same cork, with the superior end half an inch below the first tube, and the two lower branches are connected with the pulmonary artery and a by-pass respectively.

This arrangement acts as an air trap and also as a buffer to diminish the thud of each stroke, and functionates like the elastic walls of the large arteries to store up some of the energy of each systole.

There are four tubes connected with each "buffer." The first connects with the pump, the other three go to a manometer, a by-pass, and the organ respectively.

A tube tied into the pulmonary vein, and the by-pass from *B*, both deliver into one of the large test-tubes. These test-tubes have a side tube blown near their lower ends and are connected together by a piece of rubber on which is a screw clamp.

The object of this connection is to allow the blood to flow from one tube into the other when it arrives more quickly in one of the venous reservoirs. This it inevitably does, and as the resistance in the two circuits is variable, it would otherwise require constant attention to prevent the accumulation of the blood behind the principal resistance in the two circuits. In practice we always arrange the apparatus so that the flow through the lungs is in excess of the flow through the other circuit, and in consequence there is a continual flow from one venous reservoir into the other. As this is arterial flowing into venous blood it is of no moment.

The arrangement is precisely the same for the other pump, but in this case the circulation passes through the isolated organ and the manometer registers the pressure upon a revolving drum.

The pressure in either circulation can be absolutely controlled by varying the rate or throw of each pump or the resistance in each circuit by means of the by-pass.

In our experiments we employed an arrangement for automatically

recording the flow through the organ by means of a drop counter. Each drop as it falls hits the feather *F*. The impact is transferred by means of the two tambours *T* and *T′* to the lever of the latter, which writes a short vertical line upon the recording surface every time a drop falls. An ordinary goose feather is an excellent thing for this purpose; as it is not soiled by blood, there is no accumulation, but each drop bounces off into the receiver like "water off a duck's back." In order to keep the flow upon the feather sufficiently small to allow of drops, some of it was allowed to pass directly into the reservoir by means of a branch.

In addition to the pressure and the rate at which the drops fall the time was also recorded upon the smoked surface by a chronograph.

The whole circulation apparatus with the exception of the pumps and short lengths of rubber tubing is inserted in a saline bath kept at any desired temperature.

At first a variety of mechanical contrivances for oxygenating the blood were tried, but no satisfactory method was discovered. In all of them, sooner or later the blood began to froth, and once this had started, it proceeded to such an extent as to render prolonged continuation of the experiment impossible. The only means which was found to give complete satisfaction was to double the circulation and use the animal's own lungs to aërate the blood as described above.

The preparation was effected in the following manner:—A large dog was bled, the blood was defibrinated, strained and warmed to 30° C. in a stout-walled bottle with a good rubber cork pierced with three holes through which a long glass tube dipping to the bottom of the bottle, a short piece, and a mercury manometer were inserted. To the short piece of glass tube was attached a small hand pump by means of which the air in the bottle may be compressed, and the long one served as a delivery tube for the blood. Meanwhile a smaller dog was bled and then the blood vessels were filled with the defibrinated blood from the pressure bottle. Again the dog was bled, and again refilled, each successive lot of blood of the bleedings being defibrinated, strained and added to the stock in the pressure bottle. This proceeding was repeated until the washings ceased to yield fibrin. The collected blood was strained, the apparatus filled and the circulation started so as to be in readiness for connecting up to the organs.

The lungs and heart were next removed and one cannula secured in the pulmonary artery and the other in the left auricle. These with the lungs, were then, with the usual precautions against inclusion of air, connected up to one of the artificial circuits.

The organ (bowel or kidney) was then ligated off so as to leave the superior mesenteric or renal artery and vein in the pedicle. The organ was removed, a cannula secured in the artery and another in the vein and connected up with the second artificial circuit. The clamp was next removed from the arterial side and the by-pass closed down so as to raise the pressure to any chosen height. The artificial respiration enabled the chloroform to be introduced without dilution of the blood and without injury to the corpuscles, and the apparatus described by Embley[1] admitted of the percentage of chloroform vapour in the air administered being varied and determined with moderate accuracy. (6 % error.)

The blood-pressure was kept constant within a few millimetres during the whole time by adjusting the resistance in the by-pass.

Eight experiments were made and from the records curves have been drawn in which the rate of flow through the organ in drops per minute is expressed as ordinates and the time as abscissa. The time during which chloroform was admitted into the lungs is shown as a broad black line, and the percentage of chloroform in the air 2–3 % is indicated in each case, just above the broad line.

From a glance at the curve (Fig. 5) made from the record of Exp. 4 with an excised kidney, temp. 35° C., it will be seen that the inhalation

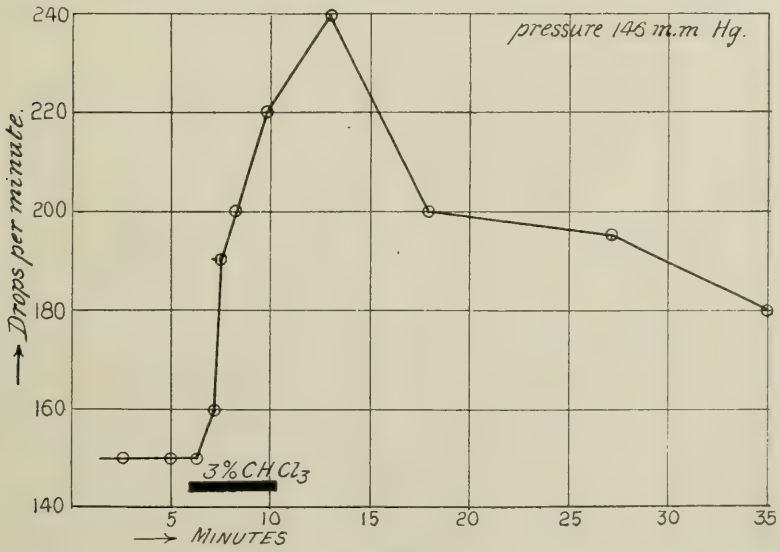

Fig. 5.

of 3 % chloroform vapour was followed within a minute by a rapid increase in flow. After four minutes the chloroform was discontinued but the increase in flow continued for another three minutes at least. The effect was considerable, viz., from 150 drops per minute before the admission of chloroform to 240 afterwards. Moreover the increased flow occasioned the excretion of about 7 c.c. of urine from the excised kidney.

After reaching its maximum, the flow diminished at first rapidly and then slowly, and 30 minutes after the commencement of the experiment had not yet returned to its previous rate. In this experiment the flow had not returned to its previous rate after one hour, when the experiment was discontinued.

Fig. 6 shows the result of 2·8 % chloroform inhaled for 11 minutes upon the flow through a portion of bowel. The general features of the curve are the same as in Experiment 4. The flow increased 50 %, and in this case returned after 50 mins. to the same amount as before the action of the chloroform.

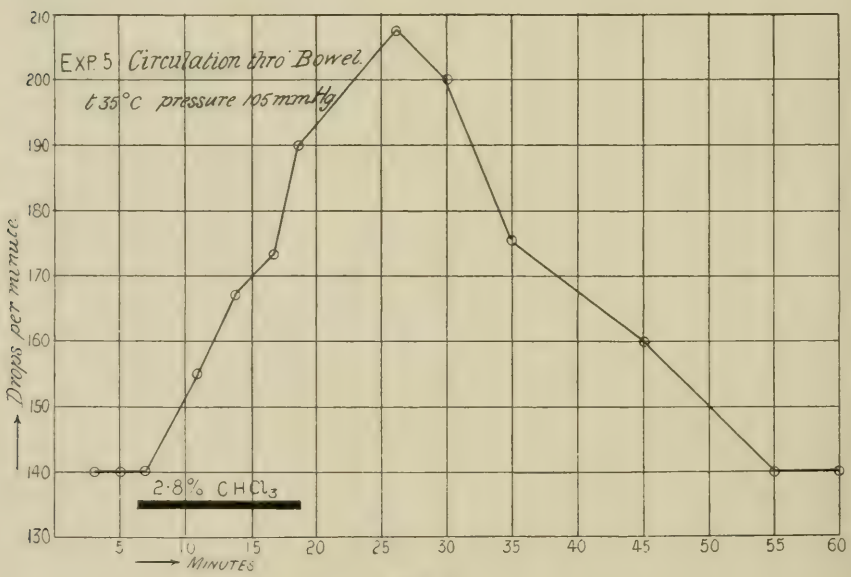

Fig. 6.

Fig. 7 is a record of another bowel experiment in which 2 % chloroform was used. In this experiment the flow was more than doubled, otherwise it shows the same features as Exps. 4 and 5.

With chloroform of about 1 % concentration the effect was much slower, but increased the flow as much as 50 per cent. in some experiments.

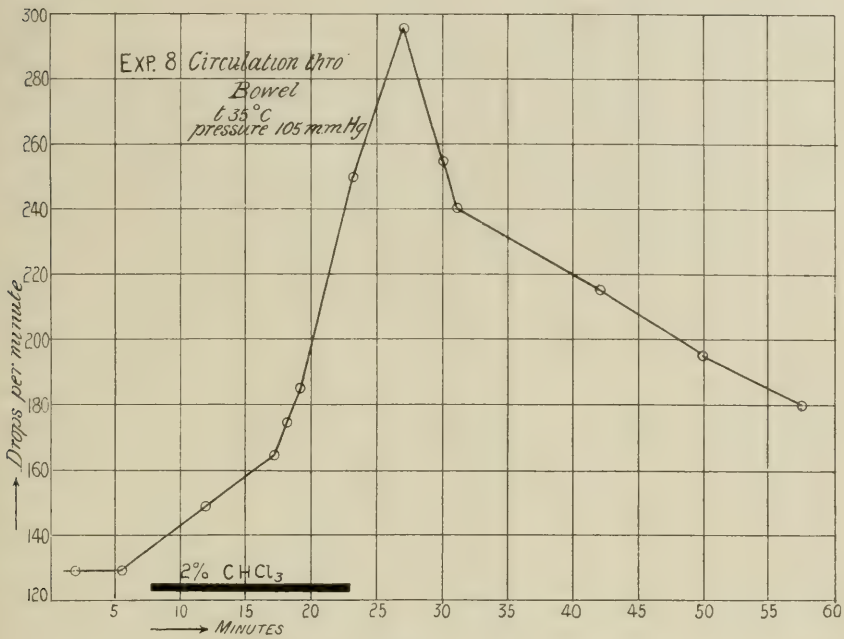

Fig. 7.

Conclusions.

From these results we conclude that the action of chloroform in the blood in such quantities as may occur with the inhalation of 1 to 3 % vapour is to directly paralyse the neuromuscular mechanism of the blood vessels of the kidney and bowel, and that a considerable part of the fall in blood-pressure which is constantly associated with the administration of chloroform may be accounted for in this manner.

That, under the conditions of our experiments, this effect upon the vessels should be more rapidly produced than is the case when chloroform of the same strength is administered to the intact animal, is only to be expected from the fact that the large reservoir capacity of the whole blood and body fluids in the latter case, is absent.

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THE CHEMICAL ACTION ON GLUCOSE OF THE LACTOSE-FERMENTING ORGANISMS OF FAECES.

By ARTHUR HARDEN.

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In a former series of experiments I found (1901) that *B. coli communis* attacks glucose in a very characteristic manner, each molecular proportion of the sugar yielding half a molecular proportion of acetic acid and of alcohol, and one molecular proportion of lactic acid, together with a small amount of succinic acid, and gaseous carbon dioxide and hydrogen. Although the amounts of the various substances produced vary somewhat with different organisms the molecular ratio of acetic acid to alcohol remains fairly constant and usually approximates to unity.

MacConkey (1905) has recently observed that the lactose-fermenting organisms of the intestine may be subdivided into four groups according to their varying action on cane-sugar and dulcite, and it was with the object of ascertaining whether these differences are correlated with any difference in the mode of attack of the organisms upon glucose that the following experiments were made.

Experimental Method. The organisms examined had all been isolated by MacConkey (1905) and the methods employed for this purpose will be found described in his paper. They were derived from ten separate samples of normal human faeces (N. H. F.), two of milk, four of water, and one each of cow-dung and horse-dung.

Each organism was cultivated in an atmosphere of nitrogen in 500 c.c. of glucose-peptone-water, containing 10 grammes of glucose, 5 grammes of Witte's peptone, and 5 grammes of chalk, for a fortnight at 37° C. The liquid was then filtered from the insoluble residue and the following analyses made.

1. The residual sugar was estimated by Pavy's method.

2. The total acid produced was estimated from the amount of calcium salt in solution, a correction being applied for dissolved calcium carbonate and calcium in the peptone.

3. Alcohol and acetic acid. The liquid was acidified with excess of oxalic acid, filtered and distilled. The first half of the distillate was collected separately, neutralised by normal caustic soda solution and again distilled, the alcohol being estimated in the distillate by the gravity method.

The total amount of volatile acids was ascertained by titrating the whole distillate, the distillation being continued in a current of steam until 100 c.c. of it only required 0·2 c.c. of normal alkali.

Formic acid, which is usually produced in small amount, was then estimated in the neutralised and evaporated solution by the mercuric chloride method, and the difference between this and the total volatile acids was taken as acetic acid.

4. The non-volatile acids were obtained from the difference between the volatile acids and the total acids produced.

5. The residue from the steam distillation was extracted with ether, the ether evaporated and the residue converted into the zinc salt, the direction of rotation of the plane of polarised light by which was then qualitatively observed.

In all 56 distinct organisms were examined and the results are embodied in the following tables. The groups into which they are divided correspond with the groups 1, 2, 3, and 4 (1) of MacConkey's provisional classification[1].

In addition, the laboratory cultures of *B. coli communis* (Escherich), *B. acidi lactici* (Hueppe), *B. lactis aerogenes* (Escherich), and *B. cloacae* (Jordan) were examined in a similar manner and the results are appended to the tables.

The sugar used is expressed in grammes; the acetic acid and alcohol in molecular proportions per molecule of sugar, and the non-volatile acids (lactic and succinic) in c c. of normal acid per gramme of sugar.

The rotation of the zinc salt was in every case found to be positive.

[1] *Loc. cit.* p. 352.

No.	Origin	Cane-sugar	Dulcite	Sugar used	Acetic acid	Alcohol	Molecular ratio $\frac{\text{alcohol}}{\text{acetic acid}}$	Non-volatile acids
	Group 1.							
19	N. H. F. i	–	–	3·78	0·43	0·61	1·43	5·79
20	,, i	–	–	3·41	0·54	0·70	1·29	5·66
21	,, ii	–	–	6·67	0·62	0·57	0·92	5·94
22	,, ii	–	–	6·65	0·65	0·63	0·96	3·79
23	,, ii	–	–	4·24	0·45	0·70	1·57	2·41
24	,, x	–	–	6·84	0·60	0·57	0·95	4·93
25	,, iii	–	–	5·85	0·68	0·66	0·98	2·86
26	,, iii	–	–	5·68	0·50	0·73	1·46	3·88
27	,, iv	–	–	4·76	0·71	0·77	1·09	2·71
28	,, iv	–	–	6·35	0·51	0·51	1·00	4·38
	Group 2.							
8	N. H. F. i	–	+	4·05	0·49	0·61	1·24	4·40
9	,, iii	–	+	5·1	0·77	0·72	0·93	2·33
10	,, iv	–	+	5·64	0·66	0·55	0·84	3·83
11	,, iv	–	+	6·13	0·55	0·55	1·00	4·65
12	,, iv	–	+	5·55	0·59	0·50	0·84	4·75
13	,, iv	–	+	5·55	0·57	0·51	0·90	4·73
14	,, iv	–	+	4·36	0·77	0·75	0·98	2·93
15	,, iv	–	+	4·78	0·82	0·75	0·93	3·89
16	,, iv	–	+	6·06	0·56	0·51	0·93	5·11
17	,, iv	–	+	5·82	0·37	0·46	0·81	6·81
18	,, iv	–	+	4·00	0·88	0·76	0·87	4·50
	Group 3.							
1	N. H. F. i	+	+	5·02	0·56	0·62	1·21	5·15
2	,, i	+	+	6·15	0·64	0·56	0·87	4·07
3	,, ii	+	+	5·59	0·71	0·71	1·00	3·02
4	,, ii	+	+	3·74	0·86	0·66	0·77	2·46
5	,, viii	+	+	4·6	0·89	0·73	0·82	2·94
6	,, viii	+	+	4·26	0·77	0·60	0·77	5·12
7	,, ix	+	+	4·81	0·75	0·53	0·71	4·16
	Group 4. Sub-group 1.							
29	Milk I	+	–	5·6	0·59	0·54	0·90	4·0
30	,,	+	–	5·00	0·60	0·59	0·98	4·85
31	,,	+	–	5·51	0·56	0·55	0·98	4·67
32	N. H. F. vi	+	–	6·21	0·81	0·73	0·90	2·23
33	,, vi	+	–	6·00	0·77	0·62	0·81	3·44
34	,, vi	+	–	6·21	0·54	0·60	1·11	3·73
35	Milk II	+	–	7·2	0·67	0·61	0·91	3·32
36	,,	+	–	6·82	0·72	0·65	0·91	3·17
37	,,	+	–	6·72	0·76	0·67	0·88	2·71
38	Horse-Dung	+	–	6·44	0·69	0·70	1·01	3·39
39	,,	+	–	6·25	0·51	0·53	1·04	5·19
40	N. H. F. vii	+	–	6·87	0·61	0·59	0·98	3·70

GROUP 4. SUB-GROUPS 2 AND 3.

No.	Origin	Cane-sugar	Dulcite	Sugar used	Acetic acid	Alcohol	Molecular ratio $\frac{\text{alcohol}}{\text{acetic acid}}$	Non-volatile acids
41	N. H. F. ii	+	–	9·52	0·05	0·77	13·4	1·20
42	,, iii	+	–	10	0·25	0·73	2·89	1·11
43	,, iii	+	–	10	0·27	0·80	2·93	1·32
44	,, iii	+	–	10	0·24	0·74	3·0	1·39
45	,, v	+	–	10	0·12	0·66	5·5	2·17
46	,, v	+	–	10	0·11	0·64	5·8	2·26
47	,, v	+	–	10	0·10	0·65	6·3	2·14
48	,, v	+	–	9·08	0·10	0·59	5·9	1·52
49	Water I	+	–	10	0·03	0·61	18	1·54
50	,, I	+	–	10	0·13	0·69	5·2	0·19
51	,, I	+	–	10	0·05	0·56	11·3	1·87
52	Water II	+	–	10	0·03	0·65	19	0·84
53	Cow-Dung	+	–	10	0·21	0·62	2·94	1·14
54	Water III	+	–	9·12	0·04	0·70	15·5	1·01
55	,, III	+	–	9·12	0·04	0·71	15·8	0·95
56	,, IV	+	–	6·13	0·12	0·65	5·11	0·77
	LABORATORY CULTURES.							
57	*B. coli com.* (Escherich)	–	+	5·57	0·58	0·64	1·12	3·67
58	*B. acidi lactici* (Hueppe)	–	–	6·37	0·54	0·54	1·0	5·29
59	*B. lactis aerogenes*	+	–	10	0·14	0·72	5·1	1·00
60	*B. cloacae*	+	–	10	0·05	0·65	13·4	0·85

As might be expected from the nature of the experiments a moderate degree of variation is found between the action of the different organisms classed here in the same group. The members of groups 1, 2, 3, and 4 (1), agree, however, in producing acetic acid and alcohol in a ratio which is not far from 1 and never exceeds 1·5 or falls below 0·7, whilst in the great majority of cases (31 out of 40) it does not differ from 1 by more than 0·2 in either direction. The average value of this ratio deduced from the 40 distinct organisms examined is 0·97. No distinctive difference capable of forming the basis of a system of classification is to be found between the action of the organisms of these four divisions.

The organisms of group 4 however are very sharply divided by this method into two classes, (*a*) Those which conform to the molecular ratio

alcohol/acetic acid = 1 ; (*b*) Those for which this ratio becomes considerably greater than 2·5.

These organisms are grouped together under the heading Group 4, Sub-groups 2 and 3, and it will be observed in the first place that the ratio of alcohol/acetic acid is always greater than 2·5, varying from about 3 to 19. In addition to this the amount of sugar used is generally large, the whole available amount being fermented in nearly every case, whilst the organisms of the other groups only use up 40–70 % of it. Finally the amount of non-volatile acids produced is much smaller than that characteristic of the other groups.

These same features are characteristic of *B. lactis aerogenes* and *B. cloacae*, the deviation being in the main due to a diminished production of acetic acid, the amount of alcohol being at the same time slightly greater. These two organisms have already been differentiated from *B. coli communis* by the amount and composition of the gas evolved (Theobald Smith, 1895). *B. lactis aerogenes* in fact appears to act on glucose in a totally different manner from the bacilli of Groups 1, 2, 3, and 4 (1). The exact nature and quantitative relations of the products formed are at present under investigation. In the cases of several of the organisms of this last group (Group 4, Sub-groups 2 and 3) the ratio alcohol/acetic acid rises from 3–5, the value characteristic of *B. lactis aerogenes*, to 11–19. The ratio given by *B. cloacae* is 13·4, and it is probable that some of these organisms belong to this or some allied type, but as a detailed chemical examination of the characteristics of such organisms has not yet been completed no definite statement can at present be made on this point.

Summary.

(1) The lactose-fermenting organisms of faeces may be divided into two groups according to the molecular ratio alcohol/acetic acid produced from glucose under specified conditions.

(*a*) Those which produce alcohol and acetic acid in approximately equal molecular proportions.

(2) Those which produce more than 2·5 molecular proportions of alcohol to 1 of acetic acid.

(3) The members of the first of these groups comprise the Groups 1, 2, 3, and 4 (1) suggested provisionally by MacConkey from the

consideration of the action of these organisms on cane-sugar and dulcite. The members of the second of the groups correspond with Group 4, Sub-groups 2 and 3, of MacConkey's provisional classification.

(4) *B. lactis aerogenes* acts upon glucose in a totally different manner from *B. coli communis* and is therefore to be regarded as a distinct organism.

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ON THE ANTITRYPTIC ACTION OF SERUM-ALBUMIN.

By S. G. HEDIN.

(*From the Lister Institute of Preventive Medicine, London.*)

It is well known that normal serum has a decided antitryptic action, which has been proved to be attached to the albumin fraction (Landsteiner[1], Cathcart[2]). On studying the effect of serum-albumin upon tryptic digestion I have found that the conditions under which the antitryptic effect appears are in many respects the same as for the antitoxins, and therefore the results may perhaps be of some general interest.

The trypsin used was prepared by digesting ox pancreas with water in presence of chloroform and toluol for two days, filtering, digesting the filtrate for some days and dialysing away the products of digestion. The serum-albumin was prepared with ammonium-sulphate and dialysed free from salt. The experiments were carried out as follows. A certain volume of a 2·5 % solution of casein in 0·1 % Na_2CO_3 was digested at 37° for 24 hours in one specimen with trypsin and antibody, and in another with the same amount of trypsin and a volume of water equal to the volume of antibody solution added to the first specimen. The amount of digestion was determined by precipitating with tannic acid and estimating the nitrogen in equal volumes of the filtrates[3]. The specimen without antibody gives more digestion than the one with antibody, and the difference corresponds to the effect of the antibody used. As antiseptic toluol was used.

In the first instance I have tried, whether the order, in which the acting substances—casein, trypsin, antibody—are mixed together has any influence upon the result of the digestion.

[1] *Centralbl. f. Bakt.* xxvii. p. 357. 1900.
[2] This *Journal*, xxxi. p. 495. 1904.
[3] This *Journal*, xxx. p. 156. 1903.

Exp. 1. The specimens contained 100 c.c. cas. + 7·5 c.c. tryps. + 5 c.c. $\left\{\begin{matrix} H_2O \\ \text{antib.} \end{matrix}\right\}$. The agents were mixed at room temperature in the order indicated for each specimen. 50 c.c. of 10 % tannic acid sol. and 100 c.c. of filtrate were taken for each estimation. The figures recorded represent the number of c.c. of $\frac{1}{10}$ n. acid required for neutralising the ammonia obtained.

Without antibody	8·55
Cas.—tryps.—antib. (added at once)	3·05
Cas.—tryps.—antib. (added after 1 hour)	3·05
Cas.—antib.—tryps. (added at once)	3·00
Cas.—antib.—tryps. (added after 1 hour)	2·9
Cas.—(tryps. + antib.) (mixed and added at once)	1·45
Cas.—(tryps. + antib.) (mixed and added after 1 hour)	0·95

Evidently it is indifferent in which order the agents are mixed as long as the trypsin and the antibody are added separately to the casein. If they are mixed before they are added to the casein the digestion is decidedly less than if they are added separately.

Exp. 2. 100 c.c. cas. + 15 c.c. tryps. + 5 c.c. $\left\{\begin{matrix} H_2O \\ \text{antib.} \end{matrix}\right\}$. Trypsin and antibody were mixed and kept at room temperature as indicated for each specimen before they were added to the casein. 50 c.c. tannic acid sol. and 100 c.c. filtrate were taken for each estimation.

Without antibody	12·1
Trypsin and antib. added separately	10·95
Tryps. + antib. mixed and added at once	9·2
,, ,, ,, ,, ,, after 15 mins.	7·4
,, ,, ,, ,, ,, ,, 30 ,,	6·4
,, ,, ,, ,, ,, ,, 1 hour	5·65
,, ,, ,, ,, ,, ,, 2 hours	4·85
,, ,, ,, ,, ,, ,, 3 ,,	4·85

Exp. 3. 250 c.c. cas. + 30 c.c. tryps. + 5 c.c. $\left\{\begin{matrix} H_2O \\ \text{antib.} \end{matrix}\right\}$. Trypsin and antibody were mixed and kept at room temperature before being added. 125 c.c. tannic acid and 300 c.c. filtrate were taken for each determination.

Without antibody	25·0
Tryps. + antib. mixed and added at once	22·8
,, ,, ,, ,, ,, after 5 mins.	20·35
,, ,, ,, ,, ,, ,, 10 ,,	19·65
,, ,, ,, ,, ,, ,, 15 ,,	19·25
,, ,, ,, ,, ,, ,, 20 ,,	18·9
,, ,, ,, ,, ,, ,, 30 ,,	18·6
,, ,, ,, ,, ,, ,, 40 ,,	17·95
,, ,, ,, ,, ,, ,, 2½ hours	17·9

Exps. 2 and 3 clearly bear out the fact, that when trypsin and antibody are mixed neutralisation of the former takes place gradually and up

to a certain endpoint. Therefore a certain amount of antibody neutralises a certain amount of trypsin. The amount neutralised depends very much upon the temperature at which the mixture is kept, as shown by the following experiments.

Exp. 4. Two specimens each containing 50 c.c. tryps. + 10 c.c. antib. were prepared and kept one at 22° and the other at 37°. After the expiration of the intervals of time recorded below, 5 c.c. of the mixtures were added to 25 c.c. cas., whereupon the resulting fluid was digested at 37° for 24 hours. 15 c.c. tannic acid and 25 c.c. filtrate were taken for each estimation.

Without antibody	16·65
Trypsin and antibody added separately	7·55

Trypsin and antibody mixed and kept before being added:

	at 22°	at 37°		at 22°	at 37°
15 mins.	5·05	3·45	1½ hours	4·25	2·65
30 ,,	lost	3·10	2 ,,	4·05	2·5
45 ,,	4·55	2·9	2½ ,,	3·85	2·35
1 hour	4·45	2·75	3 ,,	3·7	2·3

Exp. 5 was carried out with the same agents and in the same way as Exp. 4, only some days later and with less antibody. The two specimens contained 50 c.c. tryps. + 5 c.c. antib. + 5 c.c. H_2O.

Without antibody	15·65
Trypsin and antibody added separately	12

Trypsin and antibody mixed and kept before being added:

	at 20°	at 37°		at 20°	at 37°
15 mins.	9·05	6·4	2 hours	8·05	4·8
30 ,,	8·75	5·75	2½ ,,	8	4·6
1 hour	8·4	5·15	3 ,,	8	4·65
1½ hours	8·1	4·8			

At different temperatures therefore different amounts of trypsin are neutralised by the same amount of antibody, the amount of trypsin neutralised being larger for a high temperature than for a lower.

The following experiments will throw some light upon the question, whether a state of neutralisation corresponding to one temperature can be changed by changing the temperature.

Exp. 6. Two sets of specimens each containing 25 c.c. tryps. + 5 c.c. (antib. + H_2O)[1] were kept at different temperatures as recorded and after intervals of time indicated 5 c.c. of the mixtures were added to 25 c.c. of casein, whereupon the resulting fluid was digested at 37° for 24 hours and the amount of digestion tried as before. One set was mixed and kept at 0° for 2 days, then at 37° for 3 hours, and then again at 0° for 2 days.

[1] I express in this way, that if x c.c. of antibody were taken, then $(5 - x)$ c.c. of water were added.

	5 hours at 0°	1 day at 0°	2 days at 0°	then 3 hours at 37°	then again 2 days at 0°
1·25 c.c. antib.	13·35	12·65	13·05	11·55	11·75
2·5 ,,	8·3	lost	7·55	5·5	5·45
5 ,,	4·35	4·0	3·95	1·7	1·7

The other set was mixed and kept at 37° for 3 hours, and then at 0° for 4 days.

	3 hours at 37°	then 1 day at 0°	2 days at 0°	3 days at 0°	4 days at 0°
1·25 c.c. antib.	11·5	11·05	11·2	11·5	11·75
2·5 ,,	3·35	3·30	3·35	3·5	3·5
5 ,,	1·8	1·85	1·85	1·85	1·85

Exp. 7. Specimens all containing the same amount of trypsin and of antibody were kept as recorded and then their digesting power was determined as in Exp. 4.

Without antibody 18.

			1½ hours at 15°		20 hours at 0°	
	at 15°	at 37°	then at 37°	then 24 hours at 0°	then at 37°	then 24 hours at 0°
½ hour	11·0	7·5	9·7	9·7	9·5	9·55
1 ,,	10·9	6·75	9·45	9·5	9·45	9·45
1½ hours	10·75	6·45	9·3	9·55	9·35	9·25
2 ,,	10·7	6·10	9·25	9·3	9·25	9·25

From Exps. 6 and 7 the following inferences can be drawn:

Trypsin which has been neutralised at a high temperature is not activated again on lowering the temperature. A certain amount of antibody having neutralised its due amount of trypsin at a low temperature, will neutralise more if the temperature is raised. The amount of trypsin thus neutralised may reach the amount neutralised if the mixture had been kept at the higher temperature at once (Exp. 6 with 1·25 and 5 c.c. of antibody), but very often it does not reach that amount (Exp. 6 with 2·5 c.c. of antibody, Exp. 7). A mixture of trypsin and antitrypsin, kept at first at a low temperature and then at a higher, will not return to the first state of neutralisation if the temperature is lowered again.

The experiments are being continued, and the theoretical discussion of the results must be postponed till more particulars have been obtained.

SUMMARY.

1. If trypsin and antitrypsin are to be added separately to the substrate, then it is indifferent in which order they are added (Exp. 1).

2. If trypsin and antitrypsin are mixed before they are added to the substrate, then the neutralising effect of the antibody is larger than if they are added separately. The longer the mixture is kept before

being added to the substrate, the greater up to a certain point is the amount of trypsin neutralised (Exps. 1, 2, 3, 4, 5).

3. This maximum amount of trypsin, which the antibody is capable of neutralising under the conditions observed, is different for different temperatures, the antibody neutralising more trypsin at a high temperature than at a lower (Exps. 4, 5, 6, 7).

4. Trypsin neutralised at a high temperature is not even partly activated on lowering the temperature, whereas the amount of trypsin neutralised by a certain amount of antibody increases on raising the temperature.

5. The amount of trypsin neutralised when the mixture of trypsin and antitrypsin is kept at first at a low temperature and then at a higher does not always reach the amount neutralised when the mixture is kept at the higher temperature at once (Exps. 6, 7).

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OBSERVATIONS ON THE ACTION OF TRYPSIN. By S. G. HEDIN. (Two Figures in Text.)

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THE quantitative methods, which hitherto have been adopted for studying the action of trypsin, are Mett's method of measuring the progress of the digestion of coagulated proteids enclosed in capillary tubes[1], Vernon's method of determining the decrease of the volume of fibrin during digestion[2], Henry's method of estimating the increase of the electrical conductivity of the digesting fluid[3], and Weiss's method involving determination of the increase of the nitrogenous substances not precipitable on boiling with acetic acid[4].

In my experiments the digestion taken place has been measured by estimating the increase of the nitrogen not precipitable by tannic acid[5]. If sufficient of the tannic acid solution is added and if the precipitated fluid is left to stand for at least 12 hours, then an addition of more tannic acid causes no more precipitate to be formed. The amount of tannic acid necessary for complete precipitation is somewhat larger for the digested fluid than for the same fluid before the digestion, which seems to be due to the presence of albumoses. The volume of tannic acid solution added was always the same for all solutions which were to be compared. The volume of the tannic acid filtrate used for nitrogen determination has in many cases been only 10—25 c.c.; in cases where a greater accuracy was wanted such amounts of fluid were digested that 50, 100, 200, or even 400 c.c. of filtrate could be taken for N-determination; in these cases the fluid was evaporated down to 10—20 c.c. and then transferred into Kjeldahl flasks for combustion.

The trypsin used in my experiments was prepared by digesting ox pancreas with water in the presence of chloroform and toluol for about

[1] *Contribution à l'innervation de la glande soustomacale*, St Petersburg, 1889.

[2] This *Journal*, XXXVI. 404. 1901.

[3] *Compt. rend. Soc. Biol.* 1903, pp. 787, 789.

[4] *Zeitschr. f. physiol. Chem.* XL. p. 480. 1904.

[5] This *Journal*, XXX. p. 156. 1903.

two days at 37°, filtering and digesting the solution till it gave only a very weak biuret reaction, and a very slight precipitate with tannic acid. Nucleo-proteids present were removed with acetic acid and digestion products by dialysis. The resulting trypsin solution contained very little nitrogenous substance, the total nitrogen in 20 c.c. in two cases corresponding to 1·75 resp. 2·45 c.c. $\frac{1}{10}$ *N*. acid. The amount not precipitable by tannic acid corresponded to about 0·5 c.c. for 20 c.c. solution. The part of the nitrogen precipitable by tannic acid, having withstood prolonged action of trypsin, could without any appreciable error be considered as not digestible, and therefore the whole increase of non-precipitable nitrogen was considered as derived from the proteid added. The non-precipitable nitrogen of the trypsin solution was determined either separately or together with the corresponding nitrogen in the proteid of the control, which was digested without trypsin, and in the reagents used, and then the whole amount was deducted from the amount obtained from the digested fluid. The trypsin obtained in this way did not contain any trypsinogen, an extract of the mucous membrane of the intestine having no increasing effect upon its activity.

The proteids used for digestion were casein in Na_2CO_3 solution of varying strength, serum-albumin and white of egg, heated with a little Na_2CO_3 and dialysed till the alkali was removed as far as possible and Witte's peptone dialysed free from alkali. As antiseptic, toluol has been used in all my experiments, and all digestions were carried out at 37°.

The effect of the concentration of trypsin and of the time upon the rate of digestion.

Several series of experiments have been carried out, in which everything except the amount of trypsin was the same at the beginning of the digestion, and others in which only the time of digestion was varied. The influence of the amount of trypsin, the time of digestion being constant, can be represented by a curve as shown in Fig. 1 (p. 480). For the effect of the time of digestion, the amount of enzyme being the same, I find a curve of the same appearance. The general shape of the curve is the same as given by Bayliss for the influence of the time of digestion[1]. Further particulars will be obtained from the following series of experiments, by which the influence of the concentration of enzyme and of the time of digestion were tried at the same time.

[1] *Arch. d. Sc. Biol.* XI. Suppl. p. 261. 1905.

Exp. 1. The strength of the casein solution was 2·5 % in 0·25 % Na_2CO_3. Each specimen contained 25 c.c. cas. sol. + 20 c.c. (tryps. + H_2O)[1] ·20 c.c. tannic acid sol., and 40 c.c. filtrate were taken for each determination. The controls, each containing 25 c.c. cas. sol. + 20 c.c. H_2O, were digested for 1 day and 4 days.

The control digested 1 day gave N corresp. to 0·75 c.c. $\frac{1}{10}$ *N*. acid.
,, ,, 4 days ,, ,, 1·40 ,,

By interpolation one obtains:
for 2 days 0·95 ,,
,, 3 ,, 1·20 ,,

The non-precipitable nitrogen in 10 c.c. of trypsin was found to correspond to 0·1 ,,

From these figures the amount to be deducted from the nitrogen obtained directly from the experiments can easily be calculated.

C.c. of trypsin (p)	Time of dig. in days (24 hours) (t)	Obtained N in c.c. $\frac{1}{10}$ N acid	Control	N corresp. to dig. taken place (e)
0·5	1	3·65	0·75	2·90
1	1	6·55	0·75	5·80
1/2	2	6·80	0·95	5·85
1/3	3	6·90	1·20	5·70
1/4	4	7·50	1·40	6·10
2	1	11·1	0·75	10·35
2/2	2	11·7	0·95	10·75
2/3	3	12·05	1·20	10·85
2/4	4	12·15	1·40	10·75
2·5	1	13·75	0·80	12·95
2·5/2	2	14·20	0·95	13·25
2·5/3	3	14·55	1·20	13·35
2·5/4	4	14·55	1·40	13·15
5	1	20·95	0·80	20·15
5/2	2	21·40	1·00	20·40
5/3	3	21·5	1·20	20·30
5/4	4	21·3	1·40	19·90
7·5	1	25·35	0·85	24·50
7·5/2	2	25·65	1·00	24·65
7·5/3	3	25·60	1·25	24·35
7·5/4	4	25·2	1·40	23·80
10	1	27·8	0·85	26·95
10/2	2	28·8	1·00	27·8
10/3	3	28·25	1·25	27·0
10/4	4	28·4	1·45	26·95
15	1	31·9	0·90	31·0
15/2	2	32·75	1·05	31·7
15/3	3	32·3	1·25	31·05
15/4	4	32·2	1·45	30·75
20	1	35·0	0·95	34·05
20/2	2	lost	—	—
20/3	3	35·0	1·25	33·75
20/4	4	35·0	1·45	33·55

[1] I express in this way, that if x c.c. of trypsin were used, then (20 − x) c.c. of water were added.

The inferences which can be drawn from the above table are:

1. If the product of the amount of trypsin into the time of digestion is kept constant, then the effect is constant. In other words: in order to obtain the same effect with different amounts of trypsin the time of digestion must vary inversely with the trypsin. This time law holds good for all concentrations used in this experiment and at any rate up to 4 days.

2. For very small amounts of trypsin the effect is proportional to the time at the beginning of the digestion and then it drops. The effect of 0·5 c.c. of trypsin during the first days was as follows:

1	2	3	4	days.
2·9	5·85	8·65	10·75.	

The proportionality is valid for the 3 first days but not for the 4th.

3. For small amounts of trypsin and at the beginning of the digestion the effect is proportional to the amount of trypsin and for larger amounts it drops. Thus the effect during the first day was

0·5	1	2	c.c. of trypsin.
2·9	5·8	10·35	

The proportionality holds good for 0·5 and 1 c.c. of trypsin but not for 2 c.c. This regularity was also found by Bayliss.

The above rules are obvious particularly from the following series of experiments, which was carried out with larger amounts of casein but did not include so many different concentrations of the trypsin as the former series.

Exp. 2. 100 c.c. cas. + 10 c.c. (tryps. + H_2O), 75 c.c. of tannic acid sol. and 100 c.c. of the filtrate were taken for each estimation. The letters heading the columns are used in the same sense as before. Thus the column headed *e* gives the nitrogen obtained, diminished by the corresponding control.

p	*t*	*e*	*p*	*t*	*e*
1	1	4·40	4	1	16·35
			2	2	16·45
2	1	8·70	4/3	3	16·45
1	2	9·30	1	4	16·7
3	1	12·85	5	1	19·2
3/2	2	13·4	5/2	2	19·4
1	3	13·05	5/3	3	19·15
			5/4	4	19·2
			1	5	19·5

That the effect for small amounts of trypsin is proportional to the amount added is in particular borne out by the following series, in which all specimens were digested for 24 hours.

Exp. 3. 100 c.c. cas. + 10 c.c. (tryps. + H_2O), 50 c.c. tannic acid sol., 100 c.c. of filtrate.

p	e	$\frac{e}{p}$	p	e	$\frac{e}{p}$
1	2·40	2·40	6	14·85	2·48
2	4·95	2·48	7	17·35	2·48
3	7·55	2·52	8	19·10	2·39
4	9·85	2·46	9	20·60	2·29
5	12·45	2·49	10	22·75	2·28

$\frac{e}{p}$ is a constant up to 7 or 8 c.c. of trypsin and then the fall begins.

The following experiment shows that the time law holds good even for very small intervals of time.

Exp. 4. 25 c.c. cas. + 100 c.c. (tryps. + H_2O), 15 c.c. tannic acid sol., 100 c.c. filtrate.

p	t	e	p	t	e
100	15 mins.	11·35	100	1 hour	27·15
25	60 ,,	12	25	4 hours	26·6
100	30 ,,	19·15	100	1½ hours	30·05
25	2 hours	19·3	25	6 ,,	29·65

That the time law can be valid even when nearly all the proteid is digested is evident from the following series.

Exp. 5. 10 c.c. cas. + 5 c.c. (tryps. + H_2O), 10 c.c. tannic acid sol., 20 c.c. filtrate.

p	t (days)	e	p	t	e
1	1	10·75	4	1	15·7
0·5	2	10·4	2	2	15·55
2	1	13·4	5	1	16·55
1	2	13·3	2·5	2	16·4
3	1	14·85			
1·5	2	14·65			

The nitrogen of 10 c.c. of casein solution corresponded to 24·5 c.c. $\frac{1}{10}$ *N.* acid and therefore if all the casein had been digested, the nitrogen obtained would have been $24{\cdot}5 \times \frac{20}{25} = 19{\cdot}6$.

The concentration of the casein solution in the above experiments was 2·5 % dissolved in 0·25 % Na_2CO_3 solution. A larger amount of alkali might interfere with the action of the trypsin in such a way that the regularities referred to would not be obtained. With 0·5 % Na_2CO_3 I have sometimes found the effect of small amounts of trypsin with very much casein (200 c.c.) during the second day of digestion markedly larger

than during the first, and on doubling the amount of trypsin the effect may rise to more than the double in the same interval of time. In order as far as possible to diminish the amount of alkali I have dissolved freshly precipitated casein in 0·1 % Na_2CO_3 in such proportions that the solution contained 2·5 % of casein. With this amount of alkali the regularities referred to above were corroborated, but on trying the validity of the time law with large differences of the amounts of trypsin and of time, one finds that a specimen with a small amount of trypsin and long time of digestion gives an effect somewhat less than the one obtained from a specimen with more trypsin and a shorter time of digestion.

In order if possible to eliminate the disturbing influence of the alkali I have tried such proteids which can be kept in solution by aid of less alkali than the casein. Serum-albumin, white of egg, and Witte's peptone, as far as possible dialysed free from alkali, unmistakably follow the time law as formulated above. Some of the experiments are recorded below.

Serum-albumin. The serum-albumin was prepared with Am_2SO_4, dialysed, heated with some Na_2CO_3 in order to destroy its antitryptic properties and dialysed again till the alkaline reaction had disappeared. The total amount of nitrogen in 10 c.c. of albumin corresponded to 18 c.c. $\frac{1}{10}$ *N.* acid.

Exp. 1. 50 c.c. alb. + 50 c.c. (tryps. + H_2O), 25 c.c. tannic acid sol., 100 c.c. filtrate.

p	*t* (hours)	*e*	*p*	*t*	*e*
50	3	23·95	50	12	40·35
25	6	23·65	25	24	39·35
50	6	32·3	50	24	46·8
25	12	31·55	25	48	45·65

Exp. 2. The volumes used were the same as in Exp. 1.

p	*t* (days)	*e*	*p*	*t*	*e*
10	1	31·4	30	1	43·75
5	2	31·2	5	6	42·45
15	1	36·5	50	1	48·65
5	3	35·55	5	10	47·7
20	1	39·5			
5	4	38·4			

Exp. 3. 20 c.c. alb. + 10 c.c. (tryps. + H_2O), 10 c.c. tannic acid sol., 25 c.c. filtrate.

p	*t* (days)	*e*	*p*	*t*	
2	1	6·9	5	1	8·65
1	2	6·7	2·5	2	8·7
3	1	7·7	10	1	10·25
1·5	2	7·7	5	2	10·15

White of egg, heated with some Na_2CO_3 and dialysed for several days.

Exp. 1. 20 c.c. alb. + 20 c.c. (tryps. + H_2O), 10 c.c. tannic acid sol., 25 c.c. filtrate.

p	*t* (days)	*e*	*p*	*t*	*e*
10	1	9·6	20	1	11·6
5	2	9·75	10	2	11·6

Exp. 2. 25 c.c. alb. + 30 c.c. (tryps. + H_2O), 10 c.c. tannic acid sol., 50 c.c. filtrate.

p	*t* (days)	*e*	*p*	*t*	*e*
30	2	16·8	15	4	16·55
20	3	16·75	10	6	16·15

Witte's peptone, dialysed for one week. 50 c.c. pept. + 20 c.c. (tryps. + H_2O), 50 c.c. tannic acid sol., 100 c.c. filtrate.

p	*t* (days)	*e*	*p*	*t*	*e*
2	1	19·75	10	1	36·25
1	2	19·25	5	2	36
5	1	28·2	20	1	43·35
2·5	2	27·95	10	2	43·25

With regard to the above result obtained with casein, that for small amounts of trypsin and constant time of digestion the effect is proportional to the amount of trypsin, I wish to point out that this fact evidently affords a method for accurately determining the relative strength of trypsin solutions. In such experiments at least 100 c.c. of casein solution (2·5 % in 0·25 % Na_2CO_3) should be used, everything except the amount of trypsin being kept constant. If two experiments with different amounts of trypsin give the same effect for the unit of enzyme, then this represents the strength of the trypsin.

From the fact that at the beginning of the digestion the effect produced by small amounts of trypsin is proportional to the time of digestion, or that the absolute effect is the same for the same period of time, one must conclude that the enzyme does not lose in strength as long as this rule holds good. In one case it was found that a trypsin-solution in the presence of a large amount of casein in 0·25 % Na_2CO_3 kept its strength unaltered at 37° for at least 5 days. When kept at 37° with 0·25 % Na_2CO_3 without casein the same solution entirely lost its digesting power in 5 hours. Therefore the proteids exercise a protective influence upon the trypsin. This observation is in full agreement with statements made by several authors, *e.g.* Vernon[1], Bayliss and Starling[2].

With regard to the influence of the amount of trypsin upon the rate

[1] This *Journal*, xxvi. p. 412. 1901.
[2] This *Journal*, xxx. p. 71. 1903.

of digestion Pawlow using Metts tubes found that the so-called Schütz-Borrissow's law, found to be valid for pepsin, holds good also for trypsin[1]. This rule implies that the rate of digestion is proportional to the square root of the enzyme. Pawlow's results were roughly confirmed by Vernon[2]. As can be seen from the figures recorded my results are not in accordance with this rule. On the other hand my experiments fully corroborate Bayliss' results as far as small amounts of trypsin at the beginning of the digestion are concerned, for which Bayliss found effect proportional to amount of enzyme[3].

With other enzymes results similar to mine have been obtained. O'Sullivan and Tompson under certain conditions found that in order to obtain constant effects the amount of invertase must be varied inversely with the time[4]. Fuld found the time of coagulation of milk inversely proportional to the amount of rennet[5]. Vernon observed the same rule for peptone-splitting ferments[6], and C. J. Martin for the fibrin-ferments in the venoms of snakes[7]. Duclaux found the effect of invertase proportional to the time at the beginning of the action[8]; the same result was obtained by O'Sullivan and Tompson and by Armstrong[9]. H. Brown and Glindinning made the same observation for the action of diastase[10]. Armstrong observed that in the case of small amounts of invertase, the effect was proportional to the amount of enzyme[11]. Kastle and Loewenhart found the effect of lipase upon butyricester proportional to the amount of enzyme[12].

On the influence of the amount of substrate upon the rate of digestion.

In the experiments bearing on this subject everything except the amount of proteid was kept constant.

[1] *Arbeit. der Verdauungsdrüsen*, Wiesb. p. 31. 1898.
[2] This *Journal*, xxvi. p. 421. 1901.
[3] *Arch. d. Sciences Biol.* xi. Suppl. p. 261. 1905.
[4] *Journ. Chem. Soc. Trans.* lvii. 926. 1890.
[5] *Hofmeister's Beiträge*, ii. 169. 1902.
[6] This *Journal*, xxx. p. 334. 1903.
[7] *Ibid.* xxxii. p. 207. 1905.
[8] *Traité de Microbiologie*, ii. p. 137.
[9] *Proc. Roy. Soc.* lxxiii. p. 500. 1904.
[10] *Proc. Chem. Soc.* xviii. p. 43. 1902.
[11] *Proc. Roy. Soc.* lxxiii. p. 500. 1904.
[12] *Amer. Journ. of Physiol.* xxiv. p. 491. 1900.

Exp. 1. 5 c.c. tryps. + 15 c.c. (cas. + 0·25 % Na_2CO_3). Time of dig. = 16 hours; 10 c.c. tannic acid, 10 c.c. filtrate.

C.c. of Casein c	Effect e	$\frac{e}{c}$	C.c. of Casein c	Effect e	$\frac{e}{c}$
1	2·0	2·0	9	10·55	1·17
2	4·0	2·0	10	11·1	1·11
3	5·65	1·88	11	11·65	1·06
4	7·10	1·78	12	11·75	0·98
5	7·95	1·59	13	11·70	0·90
6	8·85	1·48	14	11·75	0·84
7	9·75	1·39	15	11·75	0·78
8	10·35	1·29			

From this table it is evident that the effect per unit of casein (contained in the last column) is largest for small amounts of casein and diminishes as the casein increases. For large amounts of casein the total effect was constant, which proves that in these samples the casein was present in excess. In the samples with very small amounts of casein (1 and 2 c.c.) the effect was proportional to the casein present. This does not necessarily indicate a general rule, because the digestion in those samples may have been complete, and the effect in that case must be proportional to the casein added. Therefore the following two series of experiments were carried out in which the digestion was interrupted after one hour's digestion. Special samples were digested further in order to make sure that the digestion was not completed in the first hour.

Exp. 2. 100 c.c. tryps. + 10 c.c. (cas. + 0·25 % Na_2CO_3), 10 c.c. tannic acid, 100 c.c. filtrate.

c	e	$\frac{e}{c}$	c	e	$\frac{e}{c}$
0·6	1·55	2·58	3	7·05	2·35
0·8	2·05	2·56	4	8·85	2·21
1	2·60	2·60	5	10·4	2·08
1·2	3·05	2·54	6	11·8	1·97
1·6	4·10	2·56	8	13·75	1·72
2	5·15	2·58	10	15·45	1·55

Control containing 0·6 c.c. of casein was digested for 24 hours and gave $e = 2$, corresponding to $\frac{e}{c} = 3, 5$.

Exp. 3. 100 c.c. tryps. + 20 c.c. (cas. + 0·25 % Na_2CO_3), 30 c.c. tannic acid, 125 c.c. filtrate.

c	e	$\frac{e}{c}$	c	e	$\frac{e}{c}$
0·5	2·1	4·2	10	16·15	1·62
0·8	3·35	4·19	12·5	18·2	1·46
1	3·95	3·95	15	19·4	1·29
5	11·55	2·31	17·5	20·65	1·18
7·5	13·95	1·86	20	21·55	1·08

A specimen with 1 c.c. of casein sol. gave after 24 hours' digestion $e = 5·75$.

The two last series of experiments bear clearly out the fact, that the effect per unit of casein increases as the total amount of casein diminishes and finally becomes constant.

Dialysed *serum-albumin* gives the same result, as borne out by the following experiment.

400 c.c. trypsin + 40 c.c. (alb. + H_2O). Time of dig. = ½ hour, 25 c.c. tannic acid, 400 c.c. filtrate. Total nitrogen in 10 c.c. of albumin = 18.

c	e	$\frac{e}{c}$
10	8·45	8·45
20	16·60	8·30
40	30·0	7·50

The observation that the effect per unit of proteid for small amounts of proteid becomes constant shows that for a certain amount of proteid the effect is independent of the amount of trypsin as long as the amount of trypsin exceeds a certain limit. Beyond this limit the trypsin can be considered as constant and the casein is the only variable in the digesting fluid. Therefore one might suggest that the reaction should follow the laws of a monomolecular reaction. In order to try whether this is the case Exp. 2 (p. 476) was repeated with 2 c.c. of casein solution, and the effect was determined after different intervals of time.

t (mins.)	e	t	e
15	3·9	60	4·95
30	4·45	75	5·10
45	4·8	2 days	6·7

In a monomolecular reaction the change taken place in the same interval of time is proportional to the concentration at the beginning of the time. Therefore $\frac{dC}{C}$ = const., C representing the concentration. The total amount of casein at the beginning of the digestion may be represented by the effect after complete digestion (6·7). The values of $\frac{dC}{C}$ for the subsequent intervals of 15 min. are 0·58, 0·2, 0·16, 0·08, 0·09. $\frac{dC}{C}$ therefore is no constant, the change dropping more rapidly than the concentration and consequently more rapidly than a monomolecular reaction should require. This might be explained by assuming that the different constituents of the proteid molecule are digested at a different rate, the most digestible ones yielding the nitrogen first obtained. There would be nothing surprising in this, since it is a well known fact, that the so-called antipeptone of Kühne is very slowly

digested in comparison with the other constituents of the proteid molecule; in fact it was until lately considered as quite indigestible.

As regards the influence of the amount of substrate previous investigators have made some observations with trypsin and other enzymes. Bayliss did not find any linear proportionality of the effect to the concentration of substrate in the case of casein and trypsin. A. J. Brown, experimenting with invertase and cane-sugar, found regularities similar to mine[1]. With constant amounts of invertase and small amounts of sugar the effect was proportional to the latter and within the range; where this rule was valid, he found the action of the invertase to be a monomolecular reaction, which observation lately has been confirmed by Barendrecht[2]. The proportionality of the effect to the amount of sugar with large amounts of invertase was likewise observed by Armstrong[3].

THE INFLUENCE OF THE CONCENTRATION, THE RATIO BETWEEN TRYPSIN AND SUBSTRATE BEING CONSTANT.

In the first instance I tried the effect of dilution with water, using casein dissolved in Na_2CO_3 as substrate. On dilution not only the concentration of trypsin and casein but also of the free alkali was altered and the change in the concentration of the alkali could be shown to interfere with the digestion. As a matter of fact using 2·5 % casein in 0·1 % Na_2CO_3 as substrate dilution with water in some cases produced an increase and in others a diminution of the total effect, depending upon the ratio between casein and trypsin. In one case dilution with water gave a diminution, and dilution with the same volume of 0·1 % Na_2CO_3 solution an increase of the total digestion. Therefore I tried proteids, which can be kept in solution with less alkali than casein, having removed the alkali as far as possible by dialysis. Using serum-albumin, white of egg, or Witte's peptone as substrate, it was found that the effect of digestion for equal volumes is proportional to the concentration, which of course implies that dilution with water does not affect the total effect.

The particulars of the experiments are given below. In each series of experiments the volume of the digesting fluid was constant, the concentration being different in different samples. The effect (e) is the

[1] *Proc. Chem. Soc.* XVIII. p. 14. 1902.
[2] *Zeitschr. physik. Chem.* XLIX. p. 456. 1904.
[3] *Proc. Roy. Soc.* LXXIII. p. 500. 1904.

observed number of c.c. $\frac{1}{10}$ normal acid diminished by the nitrogen obtained from the reagents. The lowest concentration used in each series represents the "unit of concentration."

Serum-albumin, prepared as pointed out p. 473. Total nitrogen in 10 c.c. corresponding to 18·5 c.c. acid.

Exp. 1. Time of dig. = 2 days, 10 c.c. tannic acid, 40 c.c. filtrate.

Concentr. (c)	e	$\frac{e}{c}$	c	e	$\frac{e}{c}$
5 alb. + 5 tryps. + 30 H_2O	3·15	3·15	15 alb. + 15 tr. + 10 H_2O	9·25	3·08
7·5 alb. + 7·5 tr. + 25 H_2O	4·75	3·17	20 alb. + 20 tr.	12·35	3·09
10 alb. + 10 tr. + 20 H_2O	6·35	3·18			

Exp. 2. 25 c.c. tannic acid, 100 c.c. filtrate.

	Time of digestion in hours					
	12		24		48	
c	e	$\frac{e}{c}$	e	$\frac{e}{c}$	e	$\frac{e}{c}$
10 alb. + 10 tr. + 80 H_2O	9·7	9·7	11·05	11·05	11·75	11·75
25 alb. + 25 tr. + 50 H_2O	23·95	9·58	27·25	10·9	29·25	11·70
50 alb. + 50 tr.	46·6	9·32	53	10·6	57·45	11·49

White of egg (p. 473). Total nitrogen in 10 c.c. = 12, 5. Time of dig. = 2 days, 20 c.c. tannic acid, 50 c.c. filtrate.

c	e	$\frac{e}{c}$	c	e	$\frac{e}{c}$
5 alb. + 2·5 tr. + 52·5 H_2O	2·95	2·95	30 alb. + 15 tr. + 15 H_2O	18·55	3·09
10 alb. + 5 tr. + 45 H_2O	5·95	2·98	40 alb. + 20 tr.	24·5	3·06
20 alb. + 10 tr. + 30 H_2O	12·30	3·08			

Witte's peptone (p. 474). Total nitrogen in 10 c.c. corresponds to 14·25. Time of dig. = 24 hours, 50 c.c. tannic acid, 100 c.c. filtrate.

c	e	$\frac{e}{c}$
5 pept. + 1 tr. + 54 H_2O	3·65	3·65
10 pept. + 2 tr. + 48 H_2O	7·5	3·75
25 pept. + 5 tr. + 30 H_2O	19·1	3·82
50 pept. + 10 tr.	39·65	3·97

Discussion of the results.

The results of the above investigations as far as the influence of the concentration of one of the acting substances is concerned, when the other is added in constant amounts, can be roughly represented by a curve as shown in Fig. 1.

The ordinates represent the amount of digestion and the abscissæ the amount of trypsin, when the amount of substrate is the same, or the amount of substrate when the amount of trypsin is the same. Since

the same effect is obtained after an equal number of *pt* (trypsin time units), the curve will be the same for constant *p* and varying *t*, as for constant *t* and varying *p*. The rise of the curve is most steep at the *origo*, showing that the effect per unit of agent is largest for small amounts, and also that the effect per unit of time is largest at the beginning of the digestion. When the amount of substrate added is the same and the amount of trypsin or the time is varied, the drop of the curve is produced in the first place by the diminution of the amount of substrate.

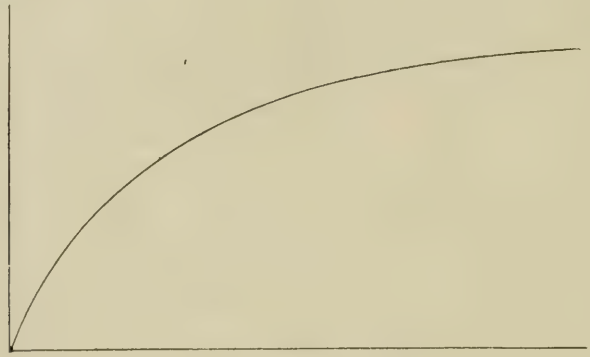

Fig. 1.

In addition to this there are some other causes for the drop, of which I wish to lay stress upon the following:

1. Some of the primary cleavage products of the proteids are more readily split into lower products than the rest (p. 477).
2. Some of the enzyme is destroyed in the course of the digestion.
3. The products of digestion exercise a checking influence upon the activity of the enzyme as pointed out for the trypsin by Bayliss[1].

That the enzymes form a sort of combination with the substrate previous to the breaking up of the same seems to be assumed by all who have of late been studying the enzymes. Armstrong adopts for the combination between invertase and cane-sugar a view similar to the one adopted by Arrhenius-Madsen for the compound between certain toxins and their antitoxins[2]. According to this view the formation of the compound enzyme-substrate is governed by the mass law,

[1] *Arch. d. Sciences Biol.* XI. Suppl. 1905.

[2] *Festskrift ved invielsen av statens seruminstitut*, Copenhagen, p. 56. 1902.

and therefore the amount formed depends upon the ratio between the agents present. Armstrong's idea is accepted by Bayliss for the trypsin-proteid compound[1]. At the beginning of my work, soon after the publication of Arrhenius-Madsen's paper, I was inclined to adopt their theory, because it seemed quite consistent with my first results, and particularly with the observation that the digestion of casein with trypsin is proportional to one of the acting substances, if the other is present in sufficient amount. The time law, or the rule that in order to obtain constant effect the time of digestion should vary inversely with the amount of trypsin added, I have however not been able to explain by aid of the mass law.

In order to find an explanation for the time law I wish first of all to point out, that different constituents of the proteid molecule are digested at a different rate. Then one only need assume that a proteid molecule of a certain kind as well as a certain constituent of a molecule always require the same number of *pt* (trypsin time units) for their digestion independently of the amount of trypsin present, and that all the different proteid molecules present as well as the constituents of the same molecule are always digested in the same order. From this it follows, that after the same number of *pt* all proteid molecules of the same kind are digested to the same extent. The digestion of a molecule can be illustrated by a sketch as shown in Fig. 2.

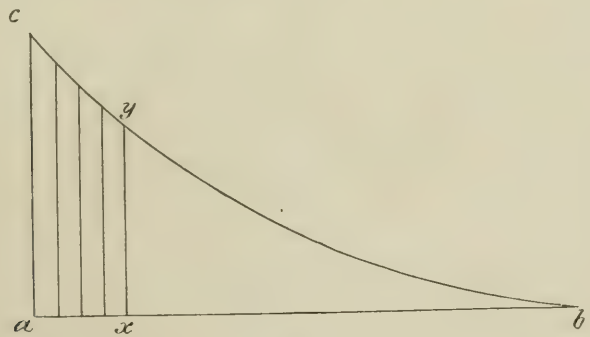

Fig. 2.

The figure represents a molecule which can be digested by means of a certain number of *pt*. The trypsin attacks the molecule at *a* and proceeds along the straight line *ab*, the length of which corresponds to

[1] *l. c.* p. 30.

the number of *pt* wanted for complete digestion. The amount of non-precipitable nitrogen produced by the successive *pt's* is represented by a small area included by a small portion of *ab*, two vertical lines and the part of the curve *ab* lying between the two lines. According to the figure therefore the amount of digestion which has taken place at the end of the 4th *pt* is represented by the area *axyc*. Evidently this area can be covered in different ways, *e.g.* by 4*p* in 1*t*, by 2*p* in 2*t*, by 1*p* in 4*t*. The curve *cb* evidently may assume any irregular shape without affecting the validity of the time law, provided the trypsin always proceeds in the same order (along the line *ab*).

The above way of explaining the validity of the time law obviously involves that the compound trypsin-substrate is formed at once for all concentrations of trypsin, or at any rate that the time required for the formation of the compound can be neglected in comparison with the time wanted for the breaking up of the substrate. This therefore must be assumed to be the case with casein, serum-albumin, white of egg, and Witte's peptone, for which the time law has been found to be valid. Furthermore the time law requires that all the trypsin present or at any rate that always the same proportion of it should be combined. This view is supported by the fact that using serum-albumin, white of egg, or Witte's peptone as substrate the same amount of trypsin and of substrate produce the same effect independently of the dilution (p. 479). For casein the effect of dilution could not be tried directly (p. 478). With a sufficient amount of casein the effect, however, was found to be proportional to the amount of trypsin, and likewise proportional to the time of digestion (p. 471), observations which are in favour of the compound trypsin-casein being formed at once and being complete.

In the above discussion it was assumed that the substrate was present in such an amount that all the trypsin was bound. According to the assumption made one molecule of proteid possesses only a certain number of affinities, where the trypsin can be fixed to it. Therefore, if so much trypsin is added that all the affinities of the substrate are saturated, then an addition of more trypsin would be of no digestive value, and the effect must be independent of the amount of trypsin. This state of things evidently corresponds to the experiments where the effect was found to be proportional to the amount of proteid added (p. 476). Upon theoretical grounds one may conclude that the time law cannot hold good if in any of the samples to be compared the trypsin is present in such an excess that some of it cannot be combined. Suppose two samples are to be digested in order to try the validity of

the time law and that the amount of proteid present in each of them contains affinities for m trypsin-units. One of the samples (A) is given $(m+n).p$ trypsin, and the digestion in this sample is interrupted after the time t, before all the substrate is digested. The other sample (B) contains p trypsin and is given the time $(m+n).t$. After the expiration of the time t the digestion in A corresponds to $m.pt$, and after the time $(m+n)t$ the digestion in B corresponds to $p(m+n)t = mpt + npt$. Therefore the digestion in the sample with less trypsin and longer time of digestion must be more advanced than in the sample with more trypsin and shorter time of digestion after the same number of trypsin time units. This is fully borne out by experiments. According to the deduction just carried out at least one of the samples to be compared should be within the range where the effect is proportional to the amount of substrate added. In order to ascertain that this is the case a series of experiments with varying amounts of substrate must be carried out and at the same time the validity of the time law should be tried for the different concentrations of the substrate. For that purpose the following series was arranged with serum-albumin as substrate.

25 c.c. tannic acid and 400 c.c. filtrate were taken for each estimation.

	Time of dig.	e	$\frac{e}{c}$
400 tryps. + 40 alb.	30 mins.	30·0	7·5
200 tr. + 40 alb. + 200 H_2O	60	30·55	7·61
400 tr. + 20 alb. + 20 H_2O	30	16·6	8·3
200 tr. + 20 alb. + 220 H_2O	60	17·35	8·68
400 tr. + 10 alb. + 30 H_2O	30	8·45	8·45
200 tr. + 10 alb. + 230 H_2O	60	9·45	9·45

Total nitrogen in 10 c.c. alb. = 18.

The figures in the last column representing the effect per unit of concentration of substrate (10 c.c.) show that of the samples with 400 c.c. trypsin those with 10 and 20 c.c. albumin gave an effect proportional to the amount of albumin, whilst the one with 40 c.c. albumin gave a lower result. Of the samples with 200 c.c. trypsin all are outside the range of proportionality. Therefore one must expect the time law to hold good for the samples with 40 c.c. alb. (difference = 1·8 %) and not for those with 20 c.c. alb. (difference = 4·5 %) and still less for those with 10 c.c. alb. (difference = 12 %). The experiments with 10 c.c. alb. were repeated in the following way.

	t	e
400 c.c. tryps. + 10 c.c. alb.	15 mins.	7·25
100 c.c. tryps. + 10 c.c. alb. + 300 c.c. H_2O	60 „	9·15

In this case the difference amounts to 26 %.

In a similar way it can, I think, be explained that in the experiments of Fuld[1] with rennet, and of Martin[2] with fibrin-ferments in the venoms of snakes, the same time law held only for moderate quantities of enzyme. I assume that the enzymes combine with the casein and fibrinogen, and in the course of a certain number of enzyme time units produce some change, in consequence of which the coagulation occurs. In the way set forth above one finds that this law cannot be valid unless all the enzyme added can be combined. If more is added then the time of clotting observed must necessarily be longer than calculated from experiments with less enzyme.

According to the time law the amount of digestion is the same in different specimens after the same number of pt, if everything except the amount of enzyme (p) and the time of digestion (t) is the same. This obviously implies that the effects of all factors which influence the digestion must be functions of pt. Therefore the diminution of digestion caused by destruction of enzyme must be the same after the same number of pt. This can easily be deducted from the time law as follows. Suppose the amounts of trypsin p and p' during the times t and t' produce the same effect. Then $\frac{p}{p'}=\frac{t'}{t}$. During the digestion the amounts of enzyme are reduced to, suppose, kp and $k'p'$. After the same intervals of time the effect must again be the same, or $\frac{kp}{k'p'}=\frac{t'}{t}$. Therefore $\frac{p}{p'}=\frac{kp}{k'p'}$ or $k=k'$.

Thus the destruction of enzyme after the same number of pt does not affect the original ratio between the amounts of enzyme.

With reference to the products of digestion it is evident that they are the same after the same number of pt, since the amount of digestion in my experiments was determined by estimating the nitrogen of the products obtained. Unless different factors influencing the digestion should happen to neutralise each other, which is not likely to be the case, one must therefore assume that the retarding effect of the products is the same during the same number of pt.

In conclusion I wish to point out that the results arrived at apply only to the proteids tried. It is proposed to extend the investigations to other proteids.

[1] *Hofmeister's Beiträge*, II. p. 169. 1902.

[2] This *Journal*, XXXII. p. 207. 1905.

Summary.

1. In order to obtain the same effect with the same amount of substrate and varying amounts of trypsin, the time of digestion should vary inversely with the amount of trypsin used, provided the substrate is present in sufficient quantity. This rule holds approximately for all the proteids tried, namely, casein, serum-albumin, white of egg and Witte's peptone.

2. If serum-albumin, white of egg, or Witte's peptone are used as substrate in as neutral solution as possible, the total effect is not affected by dilution with water, *i.e.* the effect for equal volumes is proportional to the concentration, the ratio between trypsin and substrate being constant.

3. The influence of the dilution upon the digestion of casein cannot be tried directly because of the disturbing influence of the alkali. It can however be proved that the effect is proportional to the concentration of one of the agents (casein or trypsin), if the other is present in a sufficient amount.

4. With very small amounts of trypsin and a sufficient quantity of casein the effect is proportional to the time at the very beginning of the digestion.

5. The above results may be explained as follows. All the trypsin added is at once attached to the substrate and equally distributed among all molecules of the same kind. Molecules of different proteids as well as different constituents of the same molecule may be digested at a different rate, but the same molecule or the same constituent always requires the same number of trypsin time units for its digestion, and the order in which the different molecules and the different constituents of the same molecule are digested is always the same independently of the amount of trypsin present.

6. From the time law it follows, that the influence of the destruction of enzyme and of the products of digestion must be the same after the same number of trypsin time units.

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LACTOSE-FERMENTING BACTERIA IN FAECES.

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The following experiments were begun with the object of ascertaining the distribution in nature of certain lactose-fermenting organisms which are by some grouped under the name *Bacillus coli*, but by others are regarded as belonging to a different class of organisms; a difference of opinion which is most probably the principal factor in causing the value of *B. coli* as an index of pollution to be such a vexed question among bacteriologists. The investigation, however, resolved itself into a search for the *B. lactis aerogenes* principally, and secondarily for the other lactose fermenters. Incidentally certain other points are referred to which it was thought might prove of interest though not actually pertaining to the subject of the paper.

For facility of reference the paper has been divided into sections:—

Section 1. Media—Action of potassium iodide—Methods.

Section 2. Characters of certain lactose-fermenting bacilli; Voges and Proskauer's reaction.

Section 3. Effect of environment upon biological characteristics.

Section 4. Question of preliminary incubation in a liquid medium.

Section 5. Experiments:—

(*a*) Examination of human faeces.

(*b*) Examination of animal faeces.

(*c*) Examination of milk.

(*d*) Examination of animal faeces when the animal was fed on a partially sterilised diet.

(*e*) Examination on successive occasions of a mixture of tap-water and human faeces.

Section 6. The fermentation of starch and inulin.

Section 7. Voges and Proskauer's reaction as a test for the presence of *B. lactis aerogenes* and *B. cloacae.*

Section I.

A. *Media.*

In these experiments the ordinary nutrient media were used as well as bile-salt media[1]. The basis of the latter consisted of a stock solution composed of:—

Sodium taurocholate (commercial),	0·5 gms.
Peptone (Witte),	2·0 gms.
Distilled water,	100 c.c.

For liquid media there is added to this stock solution 0·5 % of a 1 % solution of neutral-red and 0·5 % of glucose, or 1 % of the other carbohydrates or alcohols, as the case may be, and the medium is distributed into Durham's fermentation tubes[2] and sterilised in the steamer for 10 minutes on each of two days, great care being taken not to overheat the medium. If it be thought advisable white of egg may be used to clear the medium.

Bile-salt agar is made by dissolving 1·5 %—2 % agar in the stock solution. This is best done in the autoclave. The medium is cleared with white of egg and filtered. After filtration the same amount of neutral-red is added as in the case of the liquid media. Some workers add alkali to this agar but I much prefer it without, as the increased alkalinity delays the appearance of the acid reaction in the case of some organisms. Neutral-red (suggested in this connection by Grünbaum and Hume, 1902) is used as an indicator in preference to litmus because the medium is light-coloured and the differentiation much sharper. The solution of the dye should be freshly prepared, as with old solutions the result is often not so satisfactory. The addition of glycerine is to be avoided, as it seems to interfere with the sharp differentiation usually obtained. The various carbohydrates and alcohols are added, in the same proportions as for liquid media, any time after filtration, the particular kind or kinds to be added depending upon the purpose for which the agar is to be used. It is convenient to follow the plan of Drigalski and Conradi, and distribute the agar into small flasks con-

[1] *Thompson Yates Laboratories Reports,* 1900—1901.
[2] *Brit. Med. Journ.,* Vol. I. p. 138.

taining about 80 c.c., which is enough for three Petri dishes of the ordinary size. A consideration of the fermentation-reactions of the various organisms shows that by the use of certain substances, either alone or in combination, we can separate organisms by means of colour reactions. Thus, if lactose alone be added to the agar, only the lactose-fractors will produce acid and show pink colonies; if dulcite alone only the dulcite fermenters; if sorbite alone only the sorbite fermenters, and so on. This is the idea underlying all such differential media as Wurtz litmus-lactose agar, or the litmus-lactose-nutrose agar of Drigalski and Conradi. If this principle be extended by combining two or more of these carbohydrates or alcohols in the agar, we can obtain a larger number of organisms giving coloured colonies, and can thus if desirable exclude them, leaving only the colourless colonies for examination. Thus by using lactose and dulcite we include Gaertner's bacillus among the acid producers. The addition of sorbite gives an agar upon which the *Bacillus typhosus* will produce coloured colonies. Cane-sugar will exclude *B. proteus vulgaris*, and mannite one of the varieties of *B. dysenteriae*, that is, of course, on the presumption that only colourless colonies are to be taken for further investigation. It must, however, be clearly understood that it is not suggested that exactly similar results will be obtained always when working with the organisms in unknown mixtures, as in such cases we must take into account the possibility of the presence of transition forms between one group and another, and of bacilli which may have been weakened as regards their fermentative powers by unfavourable environment. Still, making allowance for both these possibilities, we can certainly lighten our labour in searching for, say, *B. dysenteriae* by excluding all those organisms in the mixture which at the time of experiment are capable of fermenting lactose, dulcite, sorbite, and cane-sugar, and if desired mannite also.

On bile-salt neutral-red lactose agar the surface colonies of *B. cloacae*, after about 20 hours in the incubator at 37° C., are usually found raised and opalescent, and some may have a red centre. With lapse of time they tend to run together into masses of mucoid-looking material with spots of deep red in them. The centres of all those colonies which remain discrete become red inside 48 hours. In 3 days the red colour may have entirely disappeared, being succeeded by a brownish-yellow colour. On a bile-salt neutral-red agar containing lactose 1 % and sorbite 0·5 % the *B. enteritidis* (Gaertner), after 24 hours' incubation at 37° C., gives a pink growth, which usually changes to brownish-yellow during the succeeding 24 hours.

With the object of stimulating the growth of *B. typhosus* bile-salt agar has been modified by the addition in varying percentages of the following substances:—Urea, asparagin, Liebig's extract, nutrose, somatose, roborat, plasmon, Nährstoff Heyden, $CaCl_2$, Na_2HPO_4, KNO_3, KI, serum, and a solution of alkaline-haematin. This latter solution was made by dissolving 7 grammes of haemoglobin in 10 c.c. of $\frac{n}{1}$ NaHO, and diluting to 100 c.c. with distilled water. Na_2HPO_4 seemed distinctly deleterious. Of the others only 5 had any apparent stimulating effect. These were:

$CaCl_2$ in the proportion of	0·034	per cent. (cf. Gabritschewsky),
Serum	1·0	,,
Alkali-haematin solution	1·0	,,
KNO_3	0·5	,,
KI	0·5	,,

B. *Action of Potassium Iodide.*

With regard to potassium iodide Elsner (1896) was the first to suggest its use in culture media. He states that his acid potato gelatin with KI 1 % inhibits most organisms except the *coli* and *typhosus* groups, and that even *B. proteus* and *B. mesentericus* do not grow well upon it. L. Grimbert (1896) disputes this statement and says that by adding non-sterilised water to tubes of Elsner's medium he obtained growth of non-pathogenic liquefying bacteria. A. Moore (1902) when using Elsner's medium found that the development of liquefying organisms frequently spoilt his plates, and to avoid this he made use of a potassium iodide potato agar, which, however, was alkaline and not acid as recommended by Elsner. Fermi (1898), tested the effect of a large number of chemical substances upon some 80 odd varieties of organisms. His stock culture medium was a neutral agar. He found *B. typhosus* would not grow in contact with more than 0·3 % potassium iodide; while *B. coli* could not withstand more than 0·75 % of the same salt.

My experiments lead me to conclude that on bile-salt lactose agar with the addition of KI 0·5 %—1 % the *B. typhosus* gives larger surface colonies than on the same agar without KI[1]. It must be remembered that the medium is alkaline to litmus and not acid, like Elsner's gelatin.

[1] The growth of *B. pestis* on bile-salt agar is also stimulated by the addition of 0·5 % KI.

A few experiments showing the effect of KI are given below:—

B. proteus vulgaris, *B. cloacae*, and *B. typhosus* were all grown in tubes of bile-salt mannite broth containing 0·5 % KI and were compared with the same organisms grown in control tubes without KI. No difference in the amount of growth as evidenced by turbidity of fluid and amount of acid or gas production could be made out.

B. typhosus was inoculated into two fermentation tubes of nutrient broth containing 2 % glucose, and to one tube 1 % KI was added and to the other 2 % KI. After 24 hours' incubation at 37° C. there was no apparent difference in the amount of growth in the bulbs, but the fluid in the closed arm of the 2 % KI tube was much clearer than that in the other tube. After 48 hours' incubation no difference could be made out between the two tubes.

Potassium iodide 0·5 % was added to broth containing 2 % glucose, and the medium was distributed into three fermentation tubes which were inoculated respectively with *B. coli communis* (Escherich), *B. capsulatus* (Pfeiffer), and *B. cloacae* (Jordan). After 3 days' incubation at 37° C., the results were:—

	Amount of gas % of closed arm	$\frac{H}{CO_2}$	Voges and Proskauer's reaction[1]
B. coli communis (Escherich)	30	$\frac{3}{2}$	–
B. capsulatus (Pfeiffer)	90	$\frac{1}{2}$	+ after 24 hours' standing.
B. cloacae (Jordan)	100	$\frac{1}{2}$	trace after 24 hours' standing. + after 48 hours' standing.

[1] See below, p. 350, hereinafter referred to as V. and P. reaction in the tables.

Nutrient broth containing 2 % glucose was filled into fermentation tubes, and 1 % KI was added. The tubes were inoculated and incubated at 37° C. for 48 hours, when the results were:—

	Amount of gas % of closed arm	$\frac{H}{CO_2}$	Voges and Proskauer's reaction
B. coli communis (Escherich)	45	$\frac{3}{2}$	–
B. lactis aerogenes (Escherich)	80	$\frac{5}{4}$	+
B. cloacae (Jordan)	100	$\frac{1}{2}$	+
B. proteus vulgaris	10	$\frac{1}{0}$	–

A broth composed of glucose 1 %, sod. taurochol. 0·5 %, peptone 2·0 %, aq. dist. 100 c.c. ± 0·5 % KI was used in a similar way. After 48 hours' incubation at 37° C., the results were:—

	Without KI			With KI 0·5 %		
	Gas amt. % of closed arm	$\frac{H}{CO_2}$	V. and P. reaction	Gas amt. % of closed arm	$\frac{H}{CO_2}$	V. and P. reaction
B. lactis aerogenes (Escherich)	40	$\frac{1}{1}$	+	70	$\frac{2}{3}$	+
B. capsulatus (Pfeiffer)	60	$\frac{2}{3}$	+	60	$\frac{1}{2}$	+
[1]T. W. F. 46	40	$\frac{1}{1}$	+	70	$\frac{2}{5}$	+
B. cloacae (Jordan)	70	$\frac{1}{2}$	+	70	$\frac{2}{5}$	+
B. coli communis (Escherich)	25	$\frac{2}{1}$	–	40	$\frac{2}{1}$	–

[1] This organism was isolated from faeces and will be referred to later in another experiment.

Just previously the same batch of broth had given:—

B. lactis aerogenes	45	$\frac{1}{1}$	+	33	$\frac{2}{1}$	+
B. cloacae	20	$\frac{1}{1}$	+	25	$\frac{1}{1}$	+

The results obtained with a similar broth but containing lactose instead of glucose were:—

	Without KI 24 hours at 37° C.			Without KI 48 hours at 37° C.			With KI 0·5 % 48 hours at 37° C.		
	Gas %	$\frac{H}{CO_2}$	V. and P. reaction	Gas %	$\frac{H}{CO_2}$	V. and P. reaction	Gas %	$\frac{H}{CO_2}$	V. and P. reaction
B. *lactis aerogenes* (Escherich)	20	$\frac{2}{5}$	+	40	$\frac{1}{1}$	+	30	$\frac{1}{2}$	+
B. capsulatus (Pfeiffer)	5	$\frac{1}{0}$	–	20	$\frac{4}{1}$	-	5	$\frac{1}{0}$	–
B. coli communis (Escherich)	10	$\frac{6}{1}$	–	20	$\frac{3}{1}$	--	10	$\frac{6}{1}$	–

In the case of *B. cloacae* the growth in the bulb of the fermentation tube was good, but in the closed arm it was scanty.

In another experiment neutral nutrient bouillon was used instead of 2 % peptone water to make a bile-salt-potassium-iodide-lactose broth. After 48 hours at 37° C.,

B. capsulatus	gave	10 %	of gas	with a ratio of	$\frac{5}{3}$	and no V. and P. reaction.
B. lactis aerogenes	,,	90	,,	,, ,,	$\frac{4}{9}$	and a marked V. and P. reaction.
B. cloacae	,,	7	,,	,, ,,	$\frac{1}{0}$	and no V. and P. reaction.
B. coli communis	,,	25	,,	,, ,,	$\frac{4}{1}$	,, ,, ,, ,,

Another tube of *B. cloacae* after 7 days' incubation showed:—

$$45\ \% \ \frac{H}{CO_2} = \frac{5}{3} \text{ V. and P. reaction marked.}$$

A broth of similar composition containing 1 % of cane-sugar as the carbohydrate, instead of glucose or lactose, yielded, after 48 hours' incubation at 37° C., the following results:—

	Without KI			With KI		
	Gas %	$\frac{H}{CO_2}$	V. and P. reaction	Gas %	$\frac{H}{CO_2}$	V. and P. reaction
B. cloacae (Jordan)	100	$\frac{1}{2}$	+	about 5	Tube broken before KHO added.	
B. capsulatus (Pfeiffer)	35	$\frac{1}{1}$	+	50	$\frac{2}{3}$	+
B. lactis aerogenes (Escherich)	30	$\frac{1}{1}$	+	40	$\frac{2}{3}$	+
B. coli communis (Escherich)	–	–	–	–	–	–
B. acidi lactici (Hüppe)	–	–	–	–	–	–
B. pneumoniae (Friedländer)	One bubble	$\frac{1}{0}$	–	One bubble	$\frac{1}{0}$	–
B. neapolitanus (Emmerich)	One bubble	$\frac{1}{0}$	–	One bubble	$\frac{1}{0}$	–
B. cloacae, inoculated from one above	75	$\frac{1}{2}$	+			

and a third trial :—

B. cloacae,						
24 hours	20					
48 hours	...	...	...	7		
48 hours	70					
3 days	90	$\frac{2}{3}$	+			
72 hours	...	...	...	14		
4 days	...	...	...	25		
5 days	...	...	...	50		
6 days	...	...	...	50	$\frac{1}{2}$	+

As Drigalski and Conradi recommend Krystall-Violett as an inhibitor of air organisms it was thought that this dye might on occasion be with advantage added to the broth. A few trials made it evident that this could be done without interfering either with the gas reaction or the production of Voges and Proskauer's reaction.

Taking these results all together it may be said :—

(1) that the addition of KI to bile-salt media may tend to delay the anaerobic growth while not interfering with multiplication aerobically; and

(2) that Voges and Proskauer's reaction is more constant than the "gas-reaction" of Theobald Smith as the former appeared in some cases when the latter was not characteristic.

C. *Methods.*

The method adopted was to emulsify a portion of the sample in a tube of bile-salt broth and to place this tube at 37° C., for, usually, 18—24 hours, but occasionally 48 hours were allowed to elapse before

plates were made. Gelatine plates were poured in the usual way, but agar plates were made after the manner of Drigalski and Conradi, *i.e.*, the melted agar was poured into the plates, allowed to solidify and dried in the warm incubator for an hour or so. A loopful of the broth culture was put on the surface of one of the plates and rubbed over it with a bent glass rod, the same rod (without recharging) being used to inoculate a second and third plate. The plates were incubated at 37° C., for 24—48 hours. Ten colonies were, as a rule, taken from each set of plates and worked through the following media:—nutrient broth, nutrient agar, nutrient gelatine, litmus-milk, and bile-salt broths containing respectively glucose, lactose, mannite, cane-sugar, and dulcite. The cultures were also tested as regards their behaviour towards Gram's method of staining. At first potato was also used and media containing glycerine, starch and inulin, but it was soon found that these media had no great value in these experiments and so their use was discontinued.

Section II.

Characters of certain Lactose-fermenting Bacilli.

As in later sections we shall be concerned with lactose-fermenting bacilli it is necessary that at the outset we should have a clear idea of the biological characters of some of the best known of these organisms, so that a comparison can be made between them and those isolated during the experiments. For this purpose I have collected the descriptions given by certain authors of the following bacilli:—

B. coli, *B. acidi lactici* (Hüppe), *B. pneumoniae* (Friedländer), *B. lactis aerogenes* (Escherich), and *B. cloacae* (Jordan).

Of these we have most knowledge concerning the *B. coli* as it has been the most carefully studied by a large number of workers. The principal characters ascribed to this organism have been gathered together in a table for ease of comparison and any special points are mentioned afterwards. The other organisms do not lend themselves to tabular description and so merely a statement has been given of the opinions expressed concerning them by the various authors mentioned.

Theobald Smith (1895) lays stress upon the amount of gas produced and upon the ratio of H to CO_2 as of value in separating *B. coli* from the *B. lactis aerogenes* and *B. cloacae*. In dextrose broth *B. coli* produces sufficient gas to fill half of the closed arm of the fermentation

TABLE A—SHOWS SOME OF THE REACTIONS ASCRIBED TO *BACILLUS COLI*[1].

	Motility	Glucose	Lactose	Cane-sugar	Milk	Indol	
Theobald Smith (1895)	+	+	+	±	A & C	...	$H : CO_2 :: 2 : 1$.
Grimbert (1896)	+	+	+	−	A & C	+	Dextrin +, mannite +, glycerine −, dulcite −.
Lembke (1896)	+	+	+	...	A & C	+	$\frac{H}{CO_2} = \frac{1+}{1}$.
Ehrenfest (1896)	+	+	+	...	A & C	+	
Orlowski (1897)	+	+	...	...	A & C	+	H_2S +.
Rothberger (1900)	±	+	...	...	A ± C	±	
Radziewsky (1900)	±	+	+	...	A + C	+	
Weissenfeld (1900)	±	+	...	...	A ± C	±	
Durham, H. E. (1900—1901) ...	+	+	+	±	A + C	±	Acts upon various other carbohydrates and alcohols.
Ford, W. W. (1901)	+	+	±	±	A + C	+	
Abbott (1902)	±	+	...	...	A + C	+	Pink colonies on litmus-lactose-agar; rarely A only in milk; growth on potato is always visible.
Muir and Ritchie (1902)	+	+	+	...	A ± C	±	
Hewlett (1902)	+	+	+	...	A + C	+	$\frac{H}{CO_2} = \frac{2}{1}$.
Winslow (1902)	...	+	...	...	A + C	+	Reduction of nitrates; agar growth not visible; reddens litmus-lactose-agar; no liquefaction of gelatine in 7 days.
Irons (1902)	...	+	+	±	A + C	...	$\frac{H}{CO_2} = \frac{1+}{1}$.
Houston, A. C. (1903)	±	+	+	±	A + C	+	
Horrocks (1903)	±	+	+	±	A + C	±	$\frac{H}{CO_2} = \frac{1}{1}$ or $\frac{1+}{1}$; acid in litmus-whey; starch unaffected; gelatine growth never wrinkled.
Jordan (1903)	+	+	+	±	A + C	±	$\frac{H}{CO_2} = \frac{2}{1}$; gelatine non-liq. in 10 days.
Brown, R. T. (1903)	+	+	+	+	A + C	+	Reduces nitrates; H_2S.
Lehmann and Neumann (1904) ...	+	+	+	±	A + C	+	
Johnson (1904)	...	+	...	...	A + C	+	Reddens litmus-lactose-agar; reduces nitrates.
Eyre (1904)	+	+	+	±	A + C	+	Reduces nitrates. Maltose, galactose, mannite, glycerine and dextrine all +.
Savage (1905)	±	+	+	±	A + C	±	

[1] Throughout these experiments the sign +, when used in connection with carbohydrates or alcohols, means that acid and gas was produced. A = acid, C = clot.

tube, the reaction of the medium is very acid, and the ratio of H to CO_2 is as 2 to 1. The acidity of 100 c.c. of bouillon = 5 c.c. of $\frac{n}{1}$ alkali, the indicator being phenol-phthalein.

Grimbert (1896) considers the property of fermenting cane-sugar is not usual among the *coli* group.

Grimbert and Legros (1900) distinguish the *coli* group from the Friedländer group by

(1) its motility,

(2) the absence of capsules in the blood of inoculated animals,

(3) the production of indol in solutions of peptone.

Rothberger (1900) thinks that the motility of this organism varies very much; the same strain being motile at one time and apparently non-motile at another. The stock bacillus of the laboratory in which he worked was a motile bacillus giving the usual growths on agar and gelatine, general turbidity in broth, gas in sugar media, feeble indol, and no clot in milk in 10 days.

Radziewsky (1900) isolated 64 *coli* from faeces by means of gelatine plates, from which he picked off only the typical "vine-leaf" colonies. The resulting cultures were plated and re-plated to ensure pure growths. He found that some of these pure cultures gave, when planted, a mixture of typical "vine-leaf" colonies and of colonies which were round, raised, and with a regular unnotched edge.

Durham (1900—1901) has suggested that because of its greater relative frequency the name *B. coli communior* should be given to that form of *B. coli* which attacks cane-sugar, and that the *B. coli communis* (Escherich) which does not ferment cane-sugar should be termed the *B. coli communis verus.* He mentions that dextrose, laevulose, mannose, arabinose, galactose, and maltose, give acid or acid and gas with races which are able to ferment dextrose. Mannite and dextrin also react like dextrose in that those bacilli which give acid or gas with one of the former will do so with the latter.

W. W. Ford (1901) also considers that the most common *B. coli* is that which possesses the power of breaking up dextrose, lactose, and cane-sugar; a second variety splits up dextrose and lactose, but not cane-sugar; and a third acts on dextrose only.

Abbott (1902) thinks that the *B. neapolitanus* of Emmerich is the same organism as the *B. coli communis* of Escherich.

Hewlett (1902) states that the *B. coli communis* has been described under the names *B. cavicida* (Brieger), and *B. neapolitanus* (Emmerich).

Horrocks (1902) describes a *B. sulcatus gasoformans*, a small, slightly motile, Gram-negative bacillus which gives growths like *B. coli* except that the surface of the gelatine growth becomes wrinkled in about five days. The gas-ratio in dextrose is about 2 to 1. The amount of acid produced in litmus-whey after 7 days at 37° C. = 20 per cent. $\frac{n}{10}$ alkali.

Orlowski (1897) is of opinion that the *B. coli* is a very variable organism. He maintains,

(1) that there are many varieties in nature;

(2) that in favourable circumstances one kind may pass over into another;

(3) that such favouring conditions may be found in the animal body, as when he injected one strain he not infrequently found another in the blood and organs, while from the intestinal contents only typical *B. coli* was isolated[1].

All these authors are agreed that *B. coli* is usually a short bacillus with rounded ends; that it does not retain the dye when stained by Gram's method; that it is a non-sporing facultative anaerobe; that the growth on agar is a greyish-white layer; that the *typical* surface colony on

[1] I trust I may be pardoned for making a slight digression here to point out that such reasoning if applied to the following cases would lead to error.

From the heart-blood of a guinea-pig which had been inoculated with a pure culture of the *B. mallei* I isolated an actively motile, short rod, Gram-negative, giving ordinary grey-white growths on agar and gelatine without liquefying the latter, general turbidity in broth, no clotting in milk but preliminary slight acidity followed by marked alkalinity, and fermenting glucose, mannite, dulcite, and sorbite with the production of acid and gas, but having no effect on lactose or cane-sugar.

On another occasion two Roux bottles of nutrient agar were inoculated with a pure culture of *B. typhosus* of which the M.L.D. (guinea-pig 250) was 0·2 c.c. of a 24 hour broth culture given intraperitoneally. After 24 hours' incubation at 37° C., the growth was swept off the surface of the agar and emulsified in 3 c.c. of 0·85 % NaCl solution. One-half of this emulsion (*i.e.* 1 Roux bottle = 15—20 agar tubes) was injected subcutaneously into a guinea-pig weighing about 250 grammes. The animal died on the fourth day and from the heart-blood was isolated in pure culture a bacillus exactly like the one described above.

A similar organism appeared to be the cause of a small epidemic which broke out amongst our stock of experimental animals. The post-mortem naked-eye appearances were those of pseudo-tuberculosis rodentium. From four stock animals examined the bacillus referred to in the two previous paragraphs was isolated in pure culture from the liver.

This suggests that organisms which morphologically and culturally are indistinguishable from the *B. enteritidis* (Gaertner) may be much more widely distributed in nature than is at present recognised. It also emphasises the importance, especially in the examination of food-stuffs, of keeping animals under observation for some time previous to experiment so as to ensure that only healthy animals are used.

gelatine is a "vine leaf," thin, filmy, translucent expansion; that gelatine is never liquefied; and that glucose is always fermented with the production of acid and gas. They differ, however, with regard to the fermentation of other carbohydrates and more particularly concerning cane-sugar. This is most probably due to the fact that organisms which give distinctly different fermentative reactions have been classed together as *B. coli communis*. But an organism can be termed *B. coli communis* (Escherich) only when it gives the constant reactions given by the original organism described by Escherich. Through the kindness of Dr H. E. Durham, who gave me a sub-culture of the original microbe, I have been able to study this bacillus closely. During the years I have had it in my possession it has varied somewhat in its morphology and in its growth on agar and gelatine, but its growth in milk and its fermentation reactions have remained constant. I, therefore, cannot agree that the *B. neapolitanus* of Emmerich is identical with the *B. coli communis* of Escherich.

It may be as well to mention here that the *B. lactis aerogenes* that I have worked with is also a sub-culture of the original strain obtained through Dr Durham.

B. acidi lactici (*Hüppe*).

Hewlett (1902) states that it is a non-liquefying, Gram-negative bacillus which produces growths something like those of *coli* but the film on gelatine is much denser.

Kruse (1903) considers the *B. acidi lactici* (Hüppe) to be identical with the *B. lactis aerogenes* (Escherich).

Prescott (1903) isolated "lactic acid bacteria" from various cereals and meals and from waters which were apparently free from all suspicion of faecal contamination and compared them with 23 varieties of *B. coli* which had been isolated from faeces or sewage. Of 64 cultures 44 gave the same cultural reactions, and of these 25 were lactic acid bacteria and 19 were *coli*. Morphologically there was no difference and all produced the same amount of acid when tested under the same conditions.

Lehmann and Neumann (1904) describe this organism as a short oval, non-motile rod, which is Gram-negative, and does not liquefy gelatine. The growth on agar resembles that of *B. coli* but is viscid. It produces general turbidity in broth, indol in peptone solutions, clotting in milk, and acid and gas in glucose and lactose. They consider it to be identical with *B. lactis aerogenes* (Escherich), and that the

B. cavicida (Brieger) and *B. neapolitanus* (Emmerich) are most closely related to it.

B. lactis aerogenes (Escherich).

Theobald Smith (1895) says that one variety of *B. lactis aerogenes*, isolated from water, is only differentiated from true *B. coli* by its want of motility. The true *B. lactis aerogenes* is differentiated by the amount of gas produced and by the composition of the gas: gas-production in glucose = 80 %—100 %, $\frac{H}{CO_2} = \frac{1}{1}$ or $\frac{1}{1+}$.

Strong (1899) discussing capsulated bacilli divides them into two groups:

Group I. *B. pneumoniae* (Friedländer), B. Wright and Mallory, *B. ozaenae, B. sputigenes crassus,* and *B. rhinoscleromatis.* Gas production: cane-sugar 50 %, glucose 30 %, lactose 30 % or maybe none. No clotting of milk.

Group II. *B. aerogenes, B. capsulatus* (Pfeiffer), *B. capsulatus* (Kruse). Gas production in all sugars about the same but most in cane-sugar. Milk is clotted.

J. Rothberger (1900) describes it as a non-motile bacillus which does not form indol. It produces general turbidity in broth, acid and clot in milk, and ferments glucose with production of acid and gas. On agar the growth is whitish and slimy, or soft and whitish with a smooth margin. The surface growth of a stab gelatine culture is grey-brown, dry and slightly irregular.

Grimbert and Legros (1900) think that *B. lactis aerogenes* (Escherich), and *B. pneumoniae* (Friedländer) are the same organism because:

(1) they are non-motile;

(2) they have capsules in the blood of inoculated animals;

(3) they do not liquefy gelatine;

(4) they do not produce indol;

(5) they ferment carbohydrates and alcohols, even though the same substances are not always attacked by both bacilli.

B. pneumoniae (Friedländer) ferments dulcite, whereas the *B. lactis aerogenes* does not.

Durham (1900—1901) places *B. lactis aerogenes* among the polysaccharide fractors, and states that it ferments dextrose, lactose, cane-sugar, and may, or may not, ferment starch and inulin. He notes that all the bacilli of this group which were tested by him gave the Voges

and Proskauer's "Kalilauge-roth-reaktion," while the bacilli of the other groups mentioned in his paper failed to do so.

Ford (1901) considers there are three forms of this organism; the most common form fermenting glucose, lactose, and cane-sugar; the second form acting on dextrose and lactose, but not on cane-sugar; and the third splitting dextrose only.

Hewlett (1902) gives the fermentation of starch and inulin as characteristics of this bacillus.

Muir and Ritchie (1902) think that this bacterium is either a variety of *B. coli* or a closely related organism. It is distinguished by its more abundant gas-production, and by its growth on gelatine and agar being thicker and whiter than that of *coli*.

Jordan (1903) describes it as a non-motile, non-liquefying bacillus which may not curdle milk until the 9th or 10th day. The gelatine colonies differ from those of *coli* in that they are less spreading, more convex and fleshy, with smooth, well-defined margins. Indol is usually produced and dextrose; lactose and cane-sugar fermented though the latter may not be acted upon.

Horrocks (1903) characterises it as a small non-motile, Gram-negative bacillus producing on agar a greyish-white tenacious growth. The gelatine colonies are thicker, and more opaque than those of *coli*. On potato the growth is coloured and gas-bubbles may appear on the surface. Milk is acidified and clotted. Indol is formed in peptone water. Glucose, lactose, cane-sugar and starch are fermented with the production of acid and gas. He also says, "Judging this organism by its power of fermenting starch, I cannot say it is frequently found in sewage polluted waters."

Paul Clairmont (1902) states that it is non-motile, gives the usual growth on agar and gelatine, does not produce indol, acidifies and clots milk, and ferments glucose, lactose, and cane-sugar.

Lehmann and Neumann (1904) consider the *B. lactis aerogenes* to be identical with the *B. acidi lactici* (Hüppe). It does not form indol, and is only differentiated from *B. coli* by its want of motility.

Taking all these opinions together the *B. lactis aerogenes* (Escherich) might be described as a non-motile, Gram-negative, non-liquefying bacillus, a facultative anaerobe, producing acid and clotting in milk, fermenting glucose, lactose, and cane-sugar and maybe also starch and inulin, and giving Voges and Proskauer's "Kalilauge-roth-reaktion" (see foot of p. 349).

B. pneumoniae (*Friedländer*).

Theobald Smith (1891) speaks of this organism as a bacillus giving general turbidity in broth, acid and clot in milk, not liquefying gelatine, and fermenting glucose, lactose, and cane-sugar. $\frac{H}{CO_2} = \frac{1+}{1}$.

Grimbert (1896) says that the Friedländer bacillus is found in water much more frequently than is generally imagined. Like *B. coli* it is Gram-negative, does not liquefy gelatine, and ferments lactose. It does not produce indol and is non-motile. He examined six strains and divided them into three groups:—

Group I. Ferments glucose, arabinose, raffinose, dextrin, mannite, maltose, cane-sugar, galactose, and lactose, but not glycerine, dulcite, or erythrite.

Group II. All these substances are decomposed except dulcite and erythrite.

Group III. Erythrite alone is not attacked.

Hewlett (1902) says the morphology is variable, the bacillus is non-motile, Gram-negative, and non-liquefying. Indol is not formed, milk is acidified and clotted. A copious, viscid, greyish growth appears on agar. On gelatine it forms a thick, white, shining, porcelain-like growth, and in stab gelatine culture the well-known "nail" growth.

Muir and Ritchie (1902) describe it as a short rod with blunt rounded ends, Gram-negative. It produces acid, with or without clot in milk, and a white, shiny, viscid growth on agar. The gelatine colonies are raised white discs. A stab culture shows the "nail" growth. Gelatine is not liquefied. Fermentation occurs in dextrose, lactose, maltose, dextrin, mannite, and may, or may not, occur in glycerine.

Clairmont (1902) gives the following characters. A non-motile bacillus giving the usual growths on agar, gelatine, and potato; general turbidity in broth; acid but no clot in milk; and fermenting glucose, lactose, and cane-sugar.

Lehmann and Neumann (1904) describe it as a short, round-ended, non-motile, non-sporing, Gram-negative rod, and a facultative anaerobe. The gelatine colonies are round, soft, white, and slimy looking. A gelatine culture is "nail-like." In broth it produces general turbidity, with a slimy deposit, and the fluid becomes somewhat viscid. Milk is acidified and usually no coagulation occurs within 20 days, but clotting may occur. The production of indol is slight. It is identical with or closely related to the *B. capsulatus* of Pfeiffer.

It would appear then that the *B. pneumoniae* (Friedländer) might be described as a non-motile, non-liquefying, Gram-negative, facultative anaerobic bacillus which produces acid in milk, with or without clotting, and ferments glucose, lactose, and cane-sugar. In the blood of experimental animals it is frequently seen to have a capsule.

B. cloacae (*Jordan*).

Theobald Smith (1895) says the *B. cloacae* is a widely-distributed, motile, strongly fermenting bacillus which gives a reaction with lactose very similar to that of *coli.* In glucose broth the gas-production amounts to 90 %—100 % of the closed arm of the fermentation tube and the ratio of H to $CO_2 = 1 : 2$ or 3.

Ford (1901) describes the typical *B. cloacae* as a motile, facultative anaerobe, fermenting dextrose, lactose, and cane-sugar, producing indol, giving general turbidity in broth, and acid and clot in milk. A second variety ferments dextrose and lactose but not cane-sugar, and does not produce indol. A third form attacks glucose only of the three sugars named, produces indol, and acidifies milk without clotting it. A fourth kind does not act upon any of the sugars, does not form indol, but clots milk. All four varieties liquefy gelatine, casein, and blood serum, and all are motile.

Irons (1902) mentions that it produces acid and clot in milk, liquefies gelatine, gives rise to acid and gas in dextrose and cane-sugar with gas ratio $H < CO_2$, and gas in lactose with a varying ratio.

Jordan (1903) writes that it is a motile bacillus fermenting dextrose and cane-sugar, which may or may not act on lactose. Indol production varies, as also does the liquefaction of blood serum and of casein. Only very rarely does it not liquefy gelatine, but the liquefaction may take 30—40 days.

Lehmann and Neumann (1904) quoting Theobald Smith (1893) describe it as a motile bacillus, liquefying gelatine, producing acid and clot in milk in 8 days, producing much gas in glucose and cane-sugar, *i.e.*, 50—95 %, $\frac{H}{CO_2} = \frac{1}{2}$. The gas-production in lactose is slow.

The *B. cloacae* (Jordan) then might be described as an actively motile, Gram-negative, facultative anaerobic bacillus, fermenting glucose and cane-sugar, and maybe lactose, producing acid and clot in milk, and which almost invariably liquefies gelatine, and may, or may not, also liquefy blood serum and casein.

The biological characters ascribed to these five organisms by the

various observers mentioned are best appreciated when tabulated for comparison :—

	Glucose	Lactose	Cane-sugar	Milk	Indol	Motility
B. coli	+	+	±	A + C[1]	+	±
B. lactis aerogenes (Escherich)	+	+	+	A + C	±	–
B. acidi lactici (Hüppe)	+	+	–	A + C	+	–
B. pneumoniae (Friedländer)	+	+	+	A ± C	∓	–
B. cloacae (Jordan)	+	±	+	A ∓ C	±	+

[1] A = Acid, C = Clot.

It is obvious that, if reliance is placed solely upon the reactions given above, these organisms may be said to be closely related; and it is not surprising that by some the *B. acidi lactici* (Hüppe) and *B. lactis aerogenes* (Escherich) should be considered identical and practically non-motile forms of the *B. coli*, or that by others the *B. lactis aerogenes* is taken to be the same organism as the *B. pneumoniae* (Friedländer). Theobald Smith differentiates these bacilli by the amount of gas produced and by the "gas ratio," and his lead is followed by most of the American bacteriologists. But these distinguishing points are not universally accepted as of value.

With the idea of, if possible, finding further points of difference I have tested the action of these and certain other organisms upon 14 carbohydrates and alcohols; have measured the amount of gas produced and the "gas-ratio," and have noted the appearance of Voges and Proskauer's reaction. The results are set out in the following table, and I desire to take this opportunity of tendering my hearty thanks to those who have so kindly sent me cultures.

It is the universal opinion that with the exception of *B. cloacae* and *B. proteus* none of these organisms liquefy gelatine, and that none of them stain by Gram's method. It has not been thought necessary to give any description of the morphology nor of the growths on agar, gelatine, or potato.

The "caustic potash red reaction[1]" was first mentioned by Voges and Proskauer (1898) in a paper on the "Bacteria of Haemorrhagic Septicaemia." They describe it thus (speaking of a Schweinepest bacillus isolated by Voges): "On the addition of caustic potash we observed a new and interesting colour reaction. If the tube be allowed to stand for 24 hours and longer at room temperature, after the addition of the potash, a beautiful fluorescent colour, somewhat similar to that of

[1] This is the reaction referred to on p. 337.

TABLE B—SHOWS CULTURAL REACTIONS OF CERTAIN GROUPS OF ORGANISMS.

Name	Obtained from	Glucose	Laevulose	Maltose	Galactose	Arabinose	Raffinose	Lactose	Cane-sugar	Mannite	Sorbite	Dulcite	Dextrin	Starch	Inulin	H in CO_2 Glucose	Voges and Proskauer reaction
B. lactis aerogenes (Escherich)	Dr H. E. Durham	+	+	+	+	+	+	+	+	+	+	–	+	±	–	$\frac{1}{1}$ or $\frac{1}{1+}$	+
B. capsulatus (Pfeiffer)	Král	+	+	+	+	+	+	+	+	+	+	–	+	±	–	,,	+
B. pneumoniae (Friedländer)	Král through Dr Bulloch	+	+	+	+	+	+	A	+	+	+	+	+	...	...	$\frac{1+}{1}$	–
,, ,, ,,	Dr H. Spitta	+	+	+	+	+	+	+	+	+	+	+	+	...	...	$\frac{1+}{1}$	–
B. neapolitanus (Emmerich)	Král	+	+	+	+	+	+	+	+	+	+	+	+	...	...	$\frac{1+}{1}$	–
B. coli communis (Escherich)	Dr H. E. Durham	+	+	+	+	+	+	+	–	+	+	+	+	–	–	$\frac{1+}{1}$	–
B. acidi lactici (Hüppe)	Král	+	+	+	+	+	+	+	–	+	+	–	+	–	–	$\frac{1+}{1}$	–
B. enteritidis (Gaertner)	Dr H. E. Durham	+	+	+	+	+	+	–	–	+	+	+	+	–	–	$\frac{1+}{1}$	–
B. icteroides (Sanarelli)	Dr Sanarelli	+	+	+	+	A	+	–	–	+	+	A	+	–	–	,,	–
B. psittacosis (Nocard)	Pasteur Institute	+	+	+	+	+	+	–	–	+	+	+	+	...	...	,,	–
B. L. (Hume)	Dr Hume	+	+	+	+	+	+	–	–	+	+	+	+	–	–	,,	–
B. epidemic jaundice	Dr H. E. Durham	+	+	+	+	+	+	–	–	+	A	?	?	–	–	,,	–
B. hog cholera (Smith, Maryland)	Dr H. de R. Morgan	+	+	+	+	A	A	–	–	+	+	A	+	...	...	,,	–
,, (Smith, Arkansas)	,,	+	+	+	+	A	A	–	–	+	+	A	+	...	...	,,	–
B. paracolon (Day)	Dr R. T. Hewlett	+	+	+	+	+	+	–	–	+	A	A	+	...	...	,,	–
B. paratyphoid (Schottmuller A)	,,	+	+	+	+	+	+	–	–	+	+	+	+	...	...	,,	–
,, (,, B)	,,	+	+	+	+	+	+	–	–	+	+	+	+	...	...	,,	–
,, (Brion & Kayser)	,,	+	+	+	+	+	+	–	–	+	+	+	+	...	...	,,	–
B. paracolon (Le Sage)	Dr H. E. Durham	+	...	+	+	+	...	–	–	+	+	+	...	–	–	,,	–
B. cloacae (Jordan)	Král	+	+	+	+	+	+	+	+	+	+	–	+	–	–	$\frac{1}{1+}$	+
B. pyogenes foetidus	Král	A	A	A	A	A	A	A	A	A	A	A	A	...	...	...	–
B. typhi abdominalis (Eberth)		A	A	A	A	–	A	–	–	A	A	–	A	–	–	...	–
B. dysenteriae (Shiga)		A	A	A	A	–	A	–	–	–	–	–	–	–	–	...	–
,, (Flexner-Gray)	Dr H. E. Durham	A	A	A	A	A	A	–	–	A	–	–	A	–	–	...	–
B. proteus vulgaris	Král	+	...	+	+	...	...	...	+	...	–	–	...	...	...	...	...
B. pseudo-tuberculosis rodentium		A	A	A	A	...	–	–	–	A	–	–	A	...	...	...	...
B. pestis		A	A	A	A	...	–	–	–	A	–	–	A	...	...	...	...

a dilute alcoholic solution of eosin, forms in the culture fluid, particularly at the open end of the tube exposed to the air. We have investigated a few of the properties of this colouring substance, which is not produced by the action of the alkali on the sugar, and have found that it is fairly resistant to the action of the external air. After a time, however, it becomes paler, and finally gives place to a dirty greenish-brown. Prolonged boiling does not affect the intensity or *nuance* of the colour. We attempted to extract the substance by means of ether and of amyl alcohol but no trace passed into these solvents. Acetic acid changes the tint into a reddish-yellow. The isolation of the colouring substance was not successful owing to the quantity present being too small. We are compelled at present to consider this new potash reaction as specific for the bacillus in question as none of the remaining bacteria, notwithstanding the variety of culture media used, gave it. Neither does the *Bacterium coli communis* give the reaction, so that we have here a further most valuable means of differentiation from these inhibitants of the intestine." The bacillus referred to as giving this reaction was one which fermented all the carbohydrates and alcohols in which it was grown, which included glucose, lactose, cane-sugar, and mannite, and thus differed from the other varieties of swine-plague and hog-cholera which have been described.

Durham (1900—1901) refers to this reaction and states that it may appear within a few hours after adding the potash (*vide supra B. lactis aerogenes*). Freeland How, junior (1904), in "Notes on *B. coli*" refers to those organisms which produce a red colour in the closed arm when standing 24 hours after the addition of potash, and says "just what produces this red colour is not known," and "if it were possible to determine the species to which a culture characterised by this colour belongs it would be of decided advantage to bacteriologists engaged in water analysis," and "the colour is destroyed by the addition of H_2SO_4 and restored by adding an alkali."

As will be seen from the table this reaction appears to obtain only in the case of the *B. lactis aerogenes* group and *B. cloacae* group, thus entirely confirming Durham's observation that it was a point of difference between the *aerogenes* group and the *coli enteritidis* and *typhosus* groups. It also helps to distinguish the *B. pneumoniae* (Friedländer) from the *B. lactis aerogenes.*

For this test it is best to use a fermentation tube, though it may be performed in an ordinary test-tube. The colour closely resembles that of a 0·2 % solution of rosolic acid in 50 % alcohol and water.

Now if we for the present neglect the question of motility and restrict our consideration to the lactose fermenting organisms solely, a glance at the table above shows that the differentiating points have reference to :—

(1) the fermentation, or otherwise, of cane-sugar and dulcite,

(2) the gas-ratio,

(3) the appearance, or non-appearance, of the "Kalilaugeroth-reaktion" of Voges and Proskauer.

And so, just for the sake of classification in these experiments, the organisms isolated have been divided arbitrarily into four groups according as they do, or do not, ferment cane-sugar and dulcite :—

Group	1	containing	these	organisms	which are	cane-sugar	–	dulcite –
„	2	„	„	„	„	–	„	+
„	3	„	„	„	„	+	„	+
„	4	„	„	„	„	+	„	–

The – sign meaning that the organism has no action, and the + sign denoting the formation of acid and gas.

Without implying that all the organisms in Group 1 are identical one may speak of them as conforming in fermentative properties to the type of *B. acidi lactici* (Hüppe). Similarly the *B. coli communis* (Escherich) may be taken as the fermentative type of Group 2, and the *B. neapolitanus* or *B. pneumoniae* (Friedländer) as representing Group 3. As regards Group 4 it will be seen from the results given below that this group may be subdivided into :—

Sub-Group	1.	? *B. coli*[1].	No liquefaction of gelatine ; absence of Voges and Proskauer reaction.
„	2.	*B. lactis aerogenes.*	No liquefaction of gelatine ; presence of Voges and Proskauer reaction.
„	3.	*B. cloacae.*	Liquefaction of gelatine or casein ; presence of Voges and Proskauer reaction.
„	4.	*Bacillus*?	Liquefying gelatine, and producing a yellow pigment.

[1] The pathogenic *Bacillus coscoroba* (*Ann. de l'Institut Pasteur*, 1900) conforms to this type.

Section III.

Effect of Environment upon Biological Characteristics.

It is not an uncommon experience in laboratory work to find that a stock organism has temporarily lost one or more of its characters, *e.g.*, indol or gas production, or the clotting of milk, and that repeated sub-cultivation soon causes the organism to resume its former vigour. These temporary enfeeblements are generally said to be due to "unfavourable environment," and it is supposed that a continuation of such unfavourable conditions may cause the change in characteristics to become permanent and thus give rise to a new variety. It might be said that Group 1 was in this way derived from the other groups, and therefore it was considered necessary to enter a little into this question. Jordan (1901) speaking of *coli* in river water says:—"It is quite possible that some bacilli may become so disguised by prolonged aquatic life as to be no longer recognisable by the methods used"; but "it is always possible to recover *coli* bacilli possessed of typical gas-producing qualities from sewage and from polluted river-waters that have been stored for some weeks in glass bottles in the laboratory." "In one instance we have found *colon* bacilli yielding typical gas production in a 1/1,000 dilution of sewage that had been standing in a bottle for 42 days, and in another case we have found them six months after the sewage had been collected."

Horrocks (1903) planted agar emulsions of *B. coli communis* (Escherich) on various samples of soils and examined the cultural reactions from time to time. The bacillus was isolated unchanged from a rich loam at the end of a month, from a virgin loam at the end of six weeks, from a virgin sandy soil at the end of 60 days. He comes to the conclusion that *B. coli* tends to die out in soil, and that the greatest duration of life is about 90 days. He also added emulsions of the same organism to various waters, which were, like the soils, kept at room temperature. From deep well-waters the *B. coli communis* was isolated unchanged after 31 days, but after 9 weeks and 3 months there was weakened indol formation and delayed coagulation of milk: from New River Company's water after 2 months with the same changes: and similarly from sterilised Thames water after 2 to 3 months. He comes to the conclusion that there is no evidence that *B. coli* ever becomes atypical.

Savage (1905), from experiments with *B. coli* and tidal mud, expresses the opinion that "None of my experiments bear out the idea that *B. coli* in mud, sterilised or unsterilised, alter some of their characters."

Remy (1900) grew *B. typhosus* and *B. coli* together in neutral bouillon. *B. typhosus* was isolated after 82 days of life in common. He says these conditions can modify profoundly the characters of the two organisms, the *B. typhosus* losing its sensibility to agglutinins and the *B. coli* its specific characters, *e.g.* gas and indol production.

Klotz (1904) in his study of an organism isolated by Adami and Chopin from tap-water and called by him *Bacillus perturbans* states that after passage through a rabbit there was a temporary loss of gas production in lactose and cane-sugar.

The object of the following experiments was to ascertain whether unsterilised laboratory tap-water would have any influence upon the *B. coli communis* (Escherich) in the direction of modification of its characters or of a shortening of its life.

A preliminary examination of the tap-water was necessary to obtain its average *coli* content. For this purpose the tap was allowed to run 5 to 10 minutes before filling a sterile stoppered bottle of about 200 c.c. capacity. Then 1 c.c. of the sample was pipetted off into a tube of bile-salt broth. On three occasions the rest of the sample was concentrated by filtration through a Berkefeld candle, the deposit being scraped off and put into a tube of bile-salt broth. On nine occasions, instead of concentrating the water by filtration, 10 c.c. of a 10 times concentrated bile-salt broth were added to the water in the bottle and the whole incubated at 37°C. for 24—48 hours, when a small quantity was used to inoculate a tube of bile-salt broth. When any of the broth tubes showed the production of acid or acid and gas, bile-salt agar plates were made and inoculated from the broth. After incubation at 37°C. for 24—48 hours a colony was taken and worked through glucose, mannite, lactose, cane-sugar, dulcite, agar, gelatine, broth and litmus milk. Twelve samples were in all examined. *B. coli* was found once out of 10 times in 1 c.c. and 8 times out of 11 in 200 c.c. It is interesting to note that *B. coli* was not found once in the deposit on the filter-candle, while it was found in almost every case when the concentrated broth was added to the rest of the sample in the bottle. It may, therefore, be accepted that the tap-water used did not usually contain *B. coli* in 1 c.c.

The *B. coli communis* (Escherich) was exposed to the influence of the tap-water in the following old way: A 24 hour broth culture was poured

into a sterile Pasteur candle, which was plugged with wool and suspended in a jar holding about $1\frac{1}{2}$—2 litres of tap-water. A similar arrangement was made for the *B. typhosus*. The jars were kept at room temperature in the dark in a cupboard. From time to time the jars were emptied and filled with fresh water, and the contents of the candles tested for the presence of *B. coli* and *B. typhosus* respectively. Thus:

3rd day.	Water changed.
5th „	„ „
6th „	*B. coli* found inside but not out.
	B. typhosus found inside but not out.
10th „	*B. coli* found inside but not out. Water changed.
19th „	Water changed.
20th „	*B. coli* and *B. typhosus* present inside the candles.
24th „	„ „ „ „ „ and water changed.
27th „	On this day gelatine plates were poured from the contents of the candles and these showed the presence of innumerable extraneous organisms.
	B. coli and *B. typhosus* found inside in one loopful.
50th „	*B. coli* and *B. typhosus* inside in 1 c.c. but not outside.
52nd „	Water changed.
77th „	*B. coli* inside but not out. *B. typhosus* apparently dead. Water changed.
81st „	No *B. typhosus* found in the whole of the contents of the filter-candle.
123rd „	*B. coli* found inside in 1 c.c.
266th „	Water changed.
267th „	*B. coli* in 1 c.c. inside.
281st „	The jar was emptied several days ago and has been allowed to remain so, and the candle to drain itself dry.
310th „	The filter-candle is empty and dry. About 300 c.c. of cold boiled distilled water poured into the filter.
311th „	*B. coli* found in 1 c.c. of the water in the filter. The fermentation of dulcite took a day or so longer than usual, otherwise the organism was unchanged.
329th „	The filter has been gradually drying. There is no water in it to-day.
357th „	10 c.c. of sterile water were poured into the filter. It was all absorbed. Then 20 c.c. more were put in, washed round and 2 c.c. used for testing. *B. coli* was recovered unchanged.
358th „	10 c.c. of sterile water were put into the filter and washed round. Then 1 c.c. was taken out and spread over the surface of a bile-salt lactose-agar plate. This was done with three plates which were incubated at 42° C.; one plate yielded 2 colonies, another 16 and the third none. One of the colonies was taken and on working out gave *B. coli* unchanged.
388th „	The filter was washed inside with 20 c.c. of sterile water, 1 c.c. of which was tested for the presence of *B. coli*. The result was negative.

The *B. coli* was not recovered from the *B. typhosus* filter in one single instance and this may, therefore, be considered as a control to the *B. coli* candle.

A further control experiment was performed as follows: The same filter-candle that had been used for the *B. coli* in the previous experiment was cleaned, plugged and sterilised. It was then suspended as before in a jar of tap-water and 10 c.c. of sterile nutrient bouillon was poured into it. The water in the jar was changed from time to time as before and the broth inside the filter tested for *B. coli*. Thus:

2nd day.	No growth whatever obtained in ordinary bouillon from 1 c.c. of the liquid inside the filter.
4th „	Water changed.
15th „	Water changed. Growth but not of *B. coli* from 1 c.c. of the liquid inside the filter.
23rd „	No *B. coli* inside.
29th „	Water changed. No *B. coli* inside.
48th „	Water changed. No *B. coli* inside.
107th „	Water changed.
125th „	No *B. coli* inside.
226th „	Water changed.
239th „	No *B. coli* inside.

The experiment was discontinued on this day as it was thought that it had been in progress for a sufficiently long time to prove that under the conditions of the experiment any *B. coli* which might be present in the tap-water would not be likely to grow through the filter and multiply to such an extent as to be present in every cubic centimetre of the inner liquid. We may conclude then that none of the *B. coli* isolated in these experiments came from the tap-water filling the jar but they were descendants of the *B. coli communis* culture originally put into the filter-candle.

It is justifiable, therefore, to assert that in this case the *B. coli communis* (Escherich) retained all its characters unchanged after an exposure of 358 days to what was a changing and unfavourable environment, a result distinctly opposed to the idea that *B. coli communis* ever becomes atypical: and tending to confirm the work of Horrocks and Savage. The result also affords presumptive evidence that one group is not derived from another by loss of characteristics on the part of some of its members.

Section IV.

Effect of Preliminary Incubation in a Liquid Medium.

In these experiments if any particular organism predominated it might be said that the results of the examination did not represent the true state, as, though all the various organisms might be present in equal numbers, the preliminary incubation in broth might allow one variety to overgrow the others. It became necessary, therefore, to make a few experiments to ascertain the effect of the preliminary incubation upon mixtures of certain known organisms.

Inoculations were, therefore, made from old gelatine cultures of *B. coli communis* (Escherich), *B. acidi lactici* (Hüppe), *B. cloacae* (Jordan), *B. lactis aerogenes* (Escherich), and C. 46 (a yellow liquefier, isolated from cow-dung) into the same tube of bile-salt mannite broth, which was incubated at 37° C. Next day surface cultivations were made on bile-salt lactose agar plates. Both broth and plates were placed at 37° C., and next day plates were again made from the broth. We have thus plates made from a mixture after 24 and 48 hours' preliminary incubation in a broth. After each set of plates had been incubated for about 24 hours, 12 colonies were taken from each set and sub-cultured in cane-sugar and dulcite. The results were :—

	B. acidi lactici	*B. coli*	*B. lactis aerogenes* *B. cloacae*	Total
24 hour plates	2	—	10 (1 was C. 46)	12
48 ,, ,,	11	1		12

The above experiment was repeated with the difference that nutrient broth cultures about one week old were used to inoculate the tube of bile-salt broth; and 10 colonies only were taken from each set of plates. Result :—

	B. acidi lactici	*B. coli*	*B. lactis aerogenes* *B. cloacae*	Total
24 hour plates	—	—	10	10
48 ,, ,,	2	—	8	10

A third experiment was made on similar lines using a mixture of *B. coli communis* and *B. acidi lactici* only. It was considered enough to take 6 colonies from each set of plates. Result :—

	B. coli	*B. acidi lactici*	Total
24 hour plates	1	5	6
48 ,, ,,	—	6	6

Further, the *B. lactis aerogenes* and *B. cloacae* were inoculated into the same tube of bile-salt mannite broth, and incubated at 37° C. At the end of 24 hours and 72 hours plates were made from the broth on bile-salt lactose agar. After 24 hours at 37° C. colonies were taken from the plates, sub-cultured into broth and examined. Results:—

	B. lactis aerogenes	*B. cloacae*	Total
24 hour plates	9	1	10
72 ,, ,,	10	0	10

A repetition of this experiment in which plates were made after the broth had been incubated at 37° C. for 24, 48, and 72 hours yielded

		B. lactis aerogenes		*B. cloacae*
24 hours	in the proportion of	4	to	1
48 ,,	,, ,,	30—40	to	1
72 ,,	,, ,,	1	to	0

The preliminary incubation might, therefore, possibly alter the relative proportions by favouring the growth of the fourth (*lactis aerogenes*) group; but if the *B. coli communis* is found, then it is not at all likely that any of the other organisms have been overwhelmed.

Section V.

As previously mentioned, these experiments were instituted for the purpose of ascertaining the relative frequency of occurrence of certain lactose-fermenting bacilli, but they resolved themselves into a search for the *B. lactis aerogenes*. The change came about in this way. My colleague, Dr A. Harden, was conducting an investigation into the chemical action of these organisms and I passed cultures of the organisms isolated over to him. As organism after organism of the fourth group (p. 352) with which I supplied him failed to satisfy his tests for a *B. lactis aerogenes* I was led to pay more attention to this organism than to others. But there was also underlying these experiments the idea that a more extended use of carbohydrates and alcohols might possibly bring to light differences between organisms of various origins. It is on this account that the experiments seem somewhat disconnected and that the references to previous work touch upon such a variety of points.

All the organisms isolated had, unless otherwise stated, the following characters in common:—

They were non-sporing, Gram-negative, facultative anaerobic bacilli,

gave grey-white growths on agar and gelatine, without liquefaction of the latter; produced acid and clotting in milk; general turbidity, with formation of indol, in broth; and fermented glucose, lactose, and mannite with the production of acid and gas. Their action on cane-sugar and dulcite is mentioned in connection with each experiment.

(*a*) *Human faeces.*

Of those who in recent years have examined human faeces there may be mentioned:—

Hans Hammerl (1897) who from his investigations came to the conclusion that whether the food is sterile or not, whether it is purely vegetable or mixed, it has no great influence upon the number of living bacteria in the faeces. The only thing noticed was that when the food was sterile the usual saprophytes commonly found in man's surroundings disappeared from the stools and almost only *B. coli* and *B. lactis aerogenes* were present and fluorescing liquefiers were absent. W. Booker (1897) studying the summer diarrhoea of infants found *B. proteus vulgaris, Streptococcus, B. coli* and *B. lactis aerogenes* to be the most common organisms. Péré (1898) mentions that the *B. coli* of sucklings attacks cane-sugar, dulcite and glycerine, whereas the *B. coli* of adults does not. Horrocks (1901) compared 80 *coli* from typhoid stools with 70 *coli* from normal faeces but could not find any constant characters which distinguished the one kind from the other. Hellström (1901) examined the faeces of new-born children, taking his samples with every aseptic precaution. He found the first meconium was sterile; later air organisms appeared; and later still these latter disappeared and were replaced by the usual intestinal organisms, especially the *B. coli* and *B. lactis aerogenes.* Ford (1901) states that *B. lactis aerogenes* is specially found in the small intestine but may pass beyond the iliocaecal valve, where, coming in contact with *B. coli*, it disappears. He also says that *B. cloacae* abounds in the caecum. Kohlbrügge (1901) considers that the small intestine either has no bacteria peculiar to itself or they belong to the *B. coli* group and that the caecum exhibits the typical *B. coli.* Brown (1903) examined the stools of 21 cases of asylum dysentery and isolated the

B. enteritidis (Gaertner)	in	1 case.
B. aerogenes		„
B. acidi lactici		„
B. xerosis		„

Houston (1902—1903) found there were more than 100 millions but less than 1,000 millions of *B. coli* per gramme of normal human faeces. He isolated and worked out the reactions of 100 of these organisms. As regards indol 99 % were positive; in milk 92 % produced acid and clotting, 8 % acid only; in dulcite 55 % gave acid and gas, while in cane-sugar media 8 % were strongly positive, 22 % feebly positive, 35 % doubtfully negative, and 35 % negative.

We might then expect to be able to isolate *B. coli* and *B. lactis aerogenes* from every sample of faeces. My own experiments do not justify this expectation as regards the latter organism as will be seen from the details of the results.

Just in case it should happen that a variation in the organisms isolated might be caused by the particular kind of broth used for the preliminary incubation, a sample of normal human faeces was taken and a loopful placed in each of 8 tubes of broth. The inoculated tubes were put into the warm incubator till the next day when gelatine plates were poured from each tube. From each set of plates 6 colonies were taken and sub-cultured. Of the 48 organisms thus isolated, 12 grew badly, or not at all, at 37° C. The remaining 36 were bacilli having the characteristics already mentioned. The action on cane-sugar and dulcite is given in the following table, which also gives the number isolated from each set of plates:

Medium used for preliminary incubation	Group I.		Group II.		Group III.		Group IV.		Total
	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	
	−	−	−	+	+	+	+	−	
Bile-salt-inulin-broth	2		3		...		...		5
,, glycerine-broth	1		2		...		...		3
,, starch ,,	1		3		...		...		4
,, dulcite ,,	...		4		...		...		4
,, cane-sugar-broth	4		—		...		...		4
,, mannite ,,	3		3		...		...		6
,, lactose ,,	2		4		...		...		6
,, glucose ,,	2		2		...		...		4
Total	15		21						36

It is noteworthy that not one of the 36 organisms fermented cane-sugar, glycerine, inulin, or starch. One produced in starch-broth an action which was of such a doubtful character that it has been considered negative. Four were distinctly motile; the rest did not show any motility under the conditions of observation. The growth on potato varied from "invisible" to an abundant yellowish-brown growth. From

this experiment it may fairly be concluded that the carbohydrate or alcohol in the medium used for preliminary incubation apparently has no selective action upon the organisms growing in it.

In the next experiment samples from the stools of persons in an ordinary state of health were examined. There were 9 samples in all taken from 3 persons; from "*A*" one sample, from "*B*" two samples, and from "*C*" six samples spread over several months. The method adopted consisted in a preliminary incubation in a bile-salt broth followed by agar plates except in the case of the 1st, 3rd, and 4th samples when gelatine plates were used. The results are set out in the following table:

Sample	Group 1.		Group 2.		Group 3.		Group 4.		Total number isolated
	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	
	−	−	−	+	+	+	+	−	
1	2		1		...		3*		6
2	12		...		...		...		12
3	...		1		2		...		3
4	2		...		2		1**		5
5	...		2		4		4		10
6	4		1		2		3		10
7	...		10		...		...		10
8	3		2		6		1		12
9	10		...		...		...		10
Total	33		17		16		12		78

* All three organisms in Group IV. were of the *B. lactis aerogenes* type.
** The organism in Group IV. was of the *B. lactis aerogenes* type.

Thus we find all four groups present in ordinary faeces with a majority in favour of the two first groups. It is striking that such a small number of the *B. lactis aerogenes* should be isolated by this method considering that the organism grows well on these media and is accepted as being so universally present in faeces. Sample No. 9 was from a liquid stool following the administration of Hunyadi-Janos Water. In the case of sample 8 plates were made after incubation periods of 1, 2, 4, and 6 hours with the idea of ascertaining whether any particular organism preponderated at these times. There was no difference to be made out between the organisms isolated from these plates and those isolated in the usual way after 18—24 hours' preliminary incubation.

The next step was to ascertain whether there was any difference in the kind and relative frequency of the organisms isolated from the stools of cases of diarrhoea or enteric fever which would enable us to

distinguish them from those of normal faeces. The method was the same as before, agar plates being made in every instance. Each sample was from a separate case.

	Group 1.		Group 2.		Group 3.		Group 4.			
Sample	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Total	Case
	−	−	−	+	+	+	+	−		
1	...		1		3		4		8	Diarrhoea; 10 organisms isolated, 2 were of the *B. pyogenes foetidus* type.
2	4		4		2		...		10	Diarrhoea.
3	...		10		...		...		10	Diarrhoea.
4	8		1		...		...		9	Convalescent from enteric fever: 1 month after commencement of attack: *B. typhosus* abundantly present.
5	8		1		1		...		10	Enteric fever.
6	1		6		3		...		10	Enteric fever.
Total	21		23		9		4		57	

These results tend to confirm those of Horrocks in showing that there is no marked difference in the character of the organisms of normal and those of diarrhoeic or enteric stools. There seems, however, a distinct preponderance of the first groups. We have again to note the absence of *B. lactis aerogenes.*

In the next experiment the faeces of one person were examined every day for a week. The diet taken during that time was ordinary mixed diet but no unboiled milk was taken. The result of the seven samples is set out below:

	Group 1.		Group 2.		Group 3.		Group 4.		
Sample	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Total number isolated
	−	−	−	+	+	+	+	−	
1	9		1		...		...		10
2	1		...		1		8		10
3	3		...		2		5		10
4	1		8		1		...		10
5	...		6		4		...		10
6	...		7		3		...		10
7	...		10		...		...		10
Total	14		32		11		13		70

In this case the only point of interest lies in the relatively small number of the first group present. This result should be considered in connection with the examination of cats' faeces (p. 367).

If we take the result of the examination of human faeces as a whole, we find that 23 samples of faeces have been analysed from which 241 lactose-fermenting organisms have been isolated, of which 83, or 34 %, belong to the first group, 93 or 38 % belong to the second group, 36 or 15 % belong to the third, and 29 or 12 % belong to the fourth. Only 4 organisms out of 241 were shown definitely to belong to the *B. lactis aerogenes* type. The *B. cloacae* was not isolated in one instance. It must be noted that the Voges and Proskauer reaction was not made use of in these experiments. The cane-sugar fermenting bacilli numbered 65 while the non-cane-sugar fermenters reached a total of 176, a proportion which is opposed to the statement that the cane-sugar fractors are the more numerous organisms.

(*b*) *Animal faeces.*

Up to the present no differentiating points have been discovered which would enable us to distinguish between the faecal bacteria of the various domesticated animals, or between these and those of human excreta, similar organisms having been found in all. Thus Smith (1891) found in swine faeces bacilli which gave general turbidity in broth, acid and clot in milk, did not liquefy gelatine, and fermented glucose, cane-sugar and lactose. The gas-reactions were similar to those of the *B. pneumoniae* (Friedländer). Dyer and Keith (1894) found no difference between human and animal *coli*. In horse-dung the most common organism was *B. equi intestinalis* which does not grow on agar at 20° C., reddens litmus-lactose-agar, and clots milk but produces no gas. Lembke (1896) examined dog faeces under varying diets, *e.g.* bread, meat, fat, and mixed diets. The experiments extended over 65 days and 81 samples of faeces were examined. Only one species of organism was always present, *i.e. B. coli*. He describes a

> *B. coli anindolicum*: a motile bacillus which produces no indol-gas in glucose 30 %; $\frac{H}{CO_2} = 5/3$; lactose 30 %, $\frac{H}{CO_2} = 4/3$; milk is acidified and clotted.

and

> *B. coli anaerogenes*: a non-motile indol-producing bacillus, acidifying and clotting milk but producing only acid in glucose and lactose.

Horrocks (1902) examined the dejecta of flies, sheep, and rabbits

and found typical *B. coli communis* to be present in all of them. He cites Moore and Wright as having examined 45 cultures of *B. coli* isolated from domesticated animals and having found 23 to be negative towards cane-sugar and 22 positive. E. Heinick (1903) examined bacteriologically the intestinal canal of 23 pigs. Of the organisms present only *B. coli* and *B. lactis aerogenes* occurred regularly. *B. coli* were the most numerous by far and were identical with human *coli*. *B. lactis aerogenes* occurred in almost equal numbers. *B. coli* were most numerous in the small gut and caecum, but were often overgrown in the rectum by *B. lactis aerogenes*. This last statement is just the contrary to Ford's conclusion concerning the fate of *lactis aerogenes* in the human caecum.

My own experiments include the examination of five samples of faeces from the horse and six from the cow and calf. The methods used were the same as in the case of human excreta and the results are given in the following tables:

Sample	Group 1. Cane-sugar −	Group 1. Dulcite −	Group 2. Cane-sugar −	Group 2. Dulcite +	Group 3. Cane-sugar +	Group 3. Dulcite +	Group 4. Cane-sugar +	Group 4. Dulcite −	Total
Horse 1	7		2		1		...		10
,, 2	4		2		4		...		10
,, 3	2		1		7		...		10
,, 4	1		2		6		2		11
,, 5	...		8		2		...		10
Total	14		15		20		2		51
Cow 1	2		...		8		...		10
,, 2	2		4		2		2		10
Calf 3	...		...		10		...		10
,, 4	3		1		2		3		9 [1]
,, 5	1		3		1		...		5
,, 6	...		4		...		Yellow liq.		4 [2]
Total	8		12		23		5		48
Total from the 11 samples	22		27		43		7		99

[1] The 10th was a *B. cloacae*.

[2] The 5th was a lactoso-fractor, liquefying gelatine and producing a yellow pigment.

In both kinds of dung the members of the third group come into greater prominence than in the case of human faeces. Beyond this there is no point of difference. The organisms give just the same reactions as those of human origin. None of the bacilli of Group 4 were identified as being of the *B. lactis aerogenes* type.

As human enteric stools had been examined there was interest in examining the faeces of animals that had been inoculated with typhoid toxine, which I was enabled to do through the kindness of Dr Allan Macfadyen.

Rabbit No. 1. Intravenous injection of sterile typhoid bacillus cell-juice. Diarrhoea, death.

Rabbit No. 2. Intravenous injection of sterile typhoid bacillus cell-juice. Diarrhoea, death in 5 days.

Rabbit No. 3. Intravenous injection of chloroformed typhoid bacillus cell-juice, death in 3 days.

	Group 1.		Group 2.		Group 3.		Group 4.		
Sample	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Total
	−	−	−	+	+	+	+	−	
No. 1	1		...		1		8		10
2	...		...		...		10		10
3	1		8		...		1		10
Total	2		8		1		19		30

An examination was also made in the case of a monkey which developed diarrhoea and died after being inoculated by Dr Macfadyen with typhoid bacillus cell-juice. The result was:

Group 1	0
,, 2	2
,, 3	1
,, 4	6
Total	9 organisms

and two other organisms which gave the reactions of *B. pyogenes foetidus*. There was isolated at the same time a short non-motile Gram-negative bacillus which gave grey-white growths on agar and gelatine without liquefaction, produced general turbidity in broth, acid only in milk, and in glucose and mannite, while lactose, cane-sugar, and dulcite were unaffected. This organism was clumped in a dilution of 1 in 200 by a dysentery serum which agglutinated its own bacillus up to 1 in 800.

(c) *Milk.*

Four samples of milk were examined with the object of ascertaining whether (1) the *B. acidi lactici* (Hüppe) or the *B. lactis aerogenes* (Escherich) was present in relatively larger numbers than the organisms of the other groups, and (2) whether, considering that milk is especially

liable to contamination by cow-dung, the lactose fermenters would be present in proportions similar to those of cow faeces. The examination was conducted in the same way as before.

Sample	Group 1. Cane-sugar	Group 1. Dulcite	Group 2. Cane-sugar	Group 2. Dulcite	Group 3. Cane-sugar	Group 3. Dulcite	Group 4. Cane-sugar	Group 4. Dulcite	Total
	−	−	−	+	+	+	+	−	
1	3		4		3		...		10
2	5		1		1		3		10
3	5		1		4		...		10
4	...		...		...		10		10
Total	13		6		8		13		40

There is nothing about this result which resembles that of the faeces of the horse or cow. It might be said almost to be just the reverse, Groups 1 and 4 predominating instead of Nos. 2 and 3.

The percentage of organisms of Group 1 is almost the same as that in human faeces but as regards Group 4 none of the organisms isolated were identified as of the *B. lactis aerogenes* type; one cannot, however, be quite certain upon this point as Voges and Proskauer's reaction was not employed.

The slight differences which appear to exist between the various materials examined are best shown by putting the percentages together in one table.

	Group 1	Group 2	Group 3	Group 4
Human faeces	34 %	39 %	15 %	12 %
Horse and cow	22	27	43	7
Milk	32·5	15	20	32·5
Typhoid (rabbit)	7	27	3	63
Cat (on special diet)	11	41	22	26

If human and animal faeces are considered together we find that some 500 organisms have been isolated of which about 24 % belong to Group 1, 37 % to Group 2, 22 % to Group 3, and 18 % to Group 4: thus showing that organisms of the *B. coli communis* type are about 30 % more numerous in faeces than any of the other groups. In this calculation I am anticipating somewhat by including the results of the examination of cat faeces.

(*d*) *Examination of animal faeces when the animal was fed on a partially sterilised diet.*

Certain rough experiments which have not been described here suggested that the presence of certain organisms in faeces might possibly

be associated with the consumption of certain food-stuffs. A cat was therefore confined in a cage and fed upon cooked food. As soon as the food had been boiled it was put while still "boiling hot" into the feeding trough. The drinking water was treated in the same way. No attempt was made to ensure absolute sterility of the food and the surroundings of the animal. Each consecutive stool was examined by the method previously used. The results are detailed below:

Sample	Group 1. Cane-sugar	Group 1. Dulcite	Group 2. Cane-sugar	Group 2. Dulcite	Group 3. Cane-sugar	Group 3. Dulcite	Group 4. Cane-sugar	Group 4. Dulcite	Total organisms isolated	
	−	−	−	+	+	+	+	−		
1	4		3		3		...		10	First stool passed after commencement of diet of boiled meat and water.
2	3		6		1		...		10	Faeces firm and dark coloured, sometimes almost black.
3	8		2		...		...		10	
4	...		...		1		9		10	
5	...		...		10		...		10	
6	...		...		10		...		10	This day diet changed to boiled milk.
7	...		...		1		9		10	First stool since change of diet, blackish-brown and very firm.
8	...		...		...		10		10	White, soft and slimy faeces.
9	...		...		2		8		10	Greyish or yellowish faeces of a soft slimy or mucoid consistency.
10	...		10		...		...		10	
11	...		9		1		...		10	
12	...		10		...		...		10	Today diet changed to boiled meat and water.
13	...		9		1		...		10	First faeces since change of diet, very firm and dark grey in colour: B. 28 added to food.
14	...		9		1		...		10	Formed greyish-brown stool.
Total	15		58		31		36		140	
15	...		1		4		5 (B. 28)		10	Pultaceous yellowish-brown faeces.
16	...		6		...		2 (B. 28)		8	

The animal was then given its freedom in the yard and put on ordinary diet. About two months later a sample was examined and yielded 10 organisms of Group 3. The animal was then again put on a diet of boiled meat and water and after a week or two of this diet a sample analysed gave 10 bacilli belonging to Group 2.

Bacillus 28 which was added to the food towards the end of the experiment was an organism of the *B. lactis aerogenes* type which had been isolated from human faeces in the earlier experiments. This organism appeared in the cat's faeces 24 hours after administration. No organisms of this type had been found in any of the previous

14 samples, but the passage of B. 28 through the intestines and its appearance in the faeces shows that if organisms of this type had been present they would have been found by the method used. Worked out in percentages the numbers read:

Group 1, 11 %	Group 2, 41 %
Group 3, 22 %	Group 4, 26 %

showing a distinct preponderance of organisms of the fermentative type of the *B. coli communis.*

The early and complete disappearance of all organisms of the first group and the entire absence of the *B. lactis aerogenes* is most remarkable.

(*e*) *Examinations at successive intervals of time, of a mixture of tap-water and human faeces.*

Bearing in mind that the *B. lactis aerogenes* grows well on bile-salt media and that it is stated to be commonly present in faeces, it is remarkable how small a number of these organisms has been isolated during the course of the previous experiments. It was thought that this might be possibly due to their being present originally in numbers which are small compared with the *B. coli,* and that they might come more into evidence if they had more time to multiply. The following experiment was performed with this idea in view and also for the purpose of ascertaining the changes, if any, which occur in the relative proportions of the four groups when faeces are diluted with water and kept for some time in a flask in the dark at room temperature. A litre flask was filled with tap-water, steamed in Koch's sterilizer for about half-an-hour, allowed to cool, and then inoculated with an amount of human faeces sufficient to cause a distinct turbidity of the whole contents of the flask. About 0·1 c.c. of the mixture was at once rubbed over the surface of three bile-salt lactose-agar plates. After 48 hours' incubation at 42° C., there was only 1 colony on the three plates. The two sterile plates were re-inoculated from the flask (= sample 1), and 0·5 c.c. from the flask was put into bile-salt mannite broth from which after 24 hours' incubation plates were made (= sample 2). On the 12th day 1 c.c. from the flask (previously well shaken) was put into the bile-salt broth and subsequently plated. This formed sample 3:—

Sample	4	was taken	on the	23rd day,
„	5	„	„	30th day,
„	6	„	„	37th day,
„	7	„	„	44th day.

In all 75 organisms were isolated and the results are shown in the following tables, the first giving the number in each group which occurred in each sample, and the second giving some details of all the organisms isolated. All the organisms fermented glucose, lactose and mannite with the formation of acid and gas. For obvious reasons more attention was paid to the members of the 4th group than to the rest.

Sample	Group 1.		Group 2.		Group 3.		Group 4.		Total number isolated
	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	Cane-sugar	Dulcite	
	−	−	−	+	+	+	+	−	
1	1		5		2		4		12
2	5		2		1		3		11
3	...		3		...		4		7
4	...		10		...		...		10
5	...		1		1		8		10
6	...		5		5		...		10
7	...		11		1		3		15
Total	6		37		10		22		75

The gas percentages and ratios (Table C, next page) were noted after 3—4 days' incubation except in the case of sample 4 when 6 days were allowed for growth. The numbers given are not necessarily those yielded by the organism when first isolated. Thus :—

No.	1st Test		2nd Test		3rd Test	
	Gas	$\frac{H}{CO_2}$	Gas	$\frac{H}{CO_2}$	Gas	$\frac{H}{CO_2}$
21	35 %	1/1	70 %	2/3	80 %	2/3
13	25	4/1	12	5/1	50	2/1
17	40	2/1	20	3/1	50	2/1
27	17	2/1	80	2/3	80	1/2
28	12	4/1	80	1/1	60	1/1
29	25	2/1	70	1/2	50	2/3
30	40	1/1	80	2/3	80	1/2
41	85	2/3	55	4/3		
42	85	2/3	60	1/1		
43	90	1/2	60	1/1		
44	80	2/3	55	1/1		
45	85	1/2	55	1/1		
46	50	3/2	50	1/1		
47	35	11/1	60	2/1		
48	45	4/1	55	3/1		
61	75	2/3	55	1/1		
62	65	2/3	40	1/1		
63	55	2/3	50	1/1		
9	80	2/3	40	1/1		
10	80	2/3	55	4/3		
11	75	1/2	55	1/1		
12	70	2/3	60	1/1		

TABLE C—SHOWING CERTAIN REACTIONS OF THE ORGANISMS ISOLATED FROM A MIXTURE OF TAP-WATER AND FAECES.

		Cane-sugar	Dulcite	Starch	Inulin	Gas %	H CO_2	Voges & Proskauer's reaction	Motility	Indol	Milk	Gelatine
Sample 1	1	–	+			25	4/1			–	A & C	Non-liq.
	2	+	+			15	4/1			–	,,	,,
	3	–	–			20	4/1			–	,,	,,
	4	–	+			25	4/1			+	,,	,,
	5	–	+			35	2/1			–	,,	,,
	6	+	+			20	4/1			–	,,	,,
	7	–	+			35	2/1			+	,,	,,
	8	–	+			25	9/1			+	,,	,,
	9	+	–	–	–	80	2/3	+	+	–	Casein liq.	Liq.
	10	+	–	–	–	80	2/3	+	+	–	,,	,,
	11	+	–	–	–	75	1/2	+	+	–	,,	,,
	12	+	–	–	–	70	1/2	+	+	–	,,	,,
Sample 2	13	+	–	–	–	25	4/1	–	–	–	A & C	Non-liq.
	14	–	–			25	12/1			+	,,	,,
	15	–	–			20	11/1			–	,,	,,
	16	–	+			20	7/1			+	,,	,,
	17	+	–	–	–	40	2/1	–	–	–	,,	,,
	18	+	+			30	2/1			–	,,	,,
	19	–	–			30	2/1				,,	,,
	20	–	+			10	1/0			–	,,	,,
	21	+	–	–	–	70	2/3	+	+	+	Casein liq.	Liq.
	22	–	–			30	4/1			–	A & C	Non-liq.
	23	–	–			23	4/1			–	,,	,,
Sample 3	24	–	+			10	1/0			+	,,	,,
	25	–	+			25	5/1			–	,,	,,
	26	–	+			10	1/0			+	,,	,,
	27	+	–	–	–	80	1/2	+	+	+	Casein liq.	Liq.
	28	+	–	–	–	80	1/2	+	+	+	,,	,,
	29	+	–	–	–	70	1/2	+	+	+	,,	,,
	30	+	–	–	–	80	1/2	+	+	–	,,	,,
Sample 4	31	–	+			20	7/1				A & C	Non-liq.
	32	–	+			20	8/1				,,	,,
	33	–	+			30	4/1				,,	,,
	34	–	+			20	7/1				,,	,,
	35	–	+			25	18/1				,,	,,
	36	–	+			20	8/1				,,	,,
	37	–	+			30	11/1				,,	,,
	38	–	+			25	4/1				,,	,,
	39	–	+			30	3/1				,,	,,
	40	–	+			25	6/1				,,	,,

Sample 5	41	+	–	–	–	85	2/3	+	+	–	Casein liq.	Liq.
	42	+	–	–	–	85	2/3	+	+	–	,,	,,
	43	+	–	–	–	90	1/2	+	+	–	,,	,,
	44	+	–	–	–	80	2/3	+	+	–	,,	,,
	45	+	–	–	–	85	1/2	+	+	+	,,	,,
	46	+	–	–	–	50	3/2	+	+	+	A & C	,,
	47	+	–	–	–	35	11/1	–	–	+	,,	Non-liq.
	48	+	–	–	–	45	4/1	–	–	–	,,	,,
	49	+	+			40	1/0			–	,,	,,
	50	–	+			40	15/1			+	,,	,,
Sample 6	51	–	+			40	4/1			+	,,	,,
	52	+	+			40	1/0			+	,,	,,
	53	–	+			22	8/1			–	,,	,,
	54	+	+			18	7/1			+	,,	,,
	55	+	+			25	4/1			+	,,	,,
	56	+	+			18	8/1			+	,,	,,
	57	+	+			25	7/1			+	,,	,,
	58	–	+			25	4/1			–	,,	,,
	59	–	+			35	5/1			+	,,	,,
	60	–	+			45	12/1			+	,,	,,
Sample 7	61	+	–	–	–	75	2/3	+	+	–	Casein liq.	Liq.
	62	+	–	–	–	65	2/3	+	+	–	,,	,,
	63	+	–	–	–	55	2/3	+	+	–	,,	,,
	64	–	+			30				+	A & C	Non-liq.
	65	–	+			40	4/1			+	,,	,,
	66	–	+			40	3/1			–	,,	,,
	67	–	+			30	6/1			+	,,	,,
	68	–	+			45	12/1			–	,,	,,
	69	–	+			45	6/1			–	,,	,,
	70	–	+			30	5/8			–	,,	,,
	71	+	+			45	8/1			+	,,	,,
	72	–	+			50	9/1			–	,,	,,
	73	–	+							+	,,	,,
	74	–	+							+	,,	,,
	75	–	+							–	,,	,,

Some organisms were tested four or more times but it is considered unnecessary to give these results. It is apparent that the gas-reaction given by these organisms may vary considerably, and that on occasion an excess of hydrogen over CO_2 is not enough to exclude a bacillus from the *cloacae* group. Voges and Proskauer's reaction, however, was given even where the gas ratio was not typical.

None of the above organisms of the fourth group liquefied blood serum in three weeks.

In litmus milk they all produced acid and caused clotting. Later, it was noticed that the surface layer of some was turning blue and the casein was being dissolved. After 3 months' growth (mostly at room temperature) the surface layer of most was blue, then a layer of turbid yellowish fluid, and at the bottom yellowish softened clot. There was distinct solution of the casein. The only exceptions were Nos. 13, 17, 46, 47, and 48. In the case of 13, 17, 47, and 48 the clot was quite firm, pink above and decolorised below. There was very little fluid. In 46 the clot was pink above and decolorised below but it was not quite so firm as the others and there was more liquid in the tube. There was not, however, any apparent solution of the casein.

In 6 % gelatine none of these organisms caused liquefaction in 5—6 weeks but when examined at the end of three months it was found that with the exception of 13, 17, 47, and 48 all had caused about half an inch of liquefaction. There was no liquefaction whatever in the case of the four bacilli mentioned. Of the 75 organisms isolated we find 22 belonging to the fourth group, and of these 18 are members of the *B. cloacae* group, one differing from the rest in not liquefying casein, and the remaining four may for the present be classed as *B. coli.* We are again struck by the absence of *B. lactis aerogenes,* and this is all the more remarkable from the fact that this sample of faeces was supplied by a person from whose dejecta *B. lactis aerogenes* had been isolated in a previous experiment. Just as in the case of cat faeces, the members of Group 1 cease to appear quite early in the experiment. After 7 weeks' exposure to the conditions of the experiment the second group still occurs in undiminished numbers.

Section VI.

Fermentation of Starch and Inulin.

The fermentation of starch or inulin appears to be a property not commonly possessed by these organisms, for 123 (60 from human, 17 from horse, and 26 from cow faeces together with 20 from milk) were tested in inulin without showing any action upon it; and of 133 (95 from human, 20 from horse, and 18 from cow faeces) only 3 showed any action on starch, and these were all of human origin.

This failure to ferment starch on the part of such a large number of organisms, coupled with the small number of bacilli of the *B. lactis aerogenes* group isolated, raised the question as to whether starch-fermenting bacteria were usually present in faeces, and suggested the advisability of carrying out one or two experiments from this point of view.

Human faeces. A loopful of human faeces was put into each of two tubes of bile-salt starch broth, which were incubated at 37° C. In each case there was evident production of acid and gas after 24 hours' growth. From these first two tubes sub-cultures were made into a second pair of starch tubes, and after a day or two from the second pair into a third pair of starch tubes. In none of these sub-cultures was there any production of acid or gas. Plates were also made from one of the first pair and from one of the second pair of tubes and three organisms isolated from each set of plates. None of these organisms caused any change in bile-salt starch broth.

Again, a small quantity of human faeces was put into a tube of ordinary broth. Twenty-four hours' incubation at 37° C. produced an excellent growth. A loopful of this mixed growth was put into bile-salt starch broth and the tube placed at 37° C. No change had occurred at the end of 5 days.

Horse faeces were put into bile-salt starch broth at 42° C. After 48 hours' growth a loopful was transferred to a second tube of starch broth. No change took place in either tube.

Cow faeces. A similar experiment was made with cow faeces with exactly the same result.

To further test this question, tubes of starch broth were inoculated as below and incubated at 37° C.

No. 1

B. lactis aerogenes *B. coli communis* *B. acidi lactici* *B. cloacae*	no fermentation in 4 days.

No. 2

B. lactis aerogenes	acid and gas in 48 hours.

No. 3

B. lactis aerogenes Horse faeces	no fermentation in 4 days.

No. 4

B. lactis aerogenes Cow faeces	? gas; a sub-culture made into starch gave a negative result.

Another experiment was made on similar lines :—

	Tube 1	2	3	4
Inoculated with	*B. lactis aerogenes*	*B. lactis aerogenes* + horse faeces	*B. lactis aerogenes* + cow faeces	*B. lactis aerogenes* + broth culture of human faeces
After 3 days at 37° C.	? any change	acid + gas	acid + gas	? any change
	Sub-cultures made into starch-broth from each tube.			
After 3 days' incubation	acid + gas	? change	? change	acid + gas
	Sub-cultures made into starch-broth from each tube.			
After 3 days' incubation	acid + gas	acid + gas	?	acid + gas

These results justify the conclusion that the fermentation of starch is a test upon which not much reliance can be placed.

Section VII.

Voges and Proskauer's reaction as a test for the presence of B. lactis aerogenes and B. cloacae.

This continued failure to isolate the *B. lactis aerogenes* raised doubt as to its constant presence in faeces, and suggested the possibility of using Voges and Proskauer's reaction as a rapid test for the presence of this organism and of *B. cloacae* in various materials, and of thus gaining an idea of the extent of their distribution in nature.

It became, therefore, necessary first to ascertain the effect of growth in mixtures upon the appearance of this reaction. For this purpose the following experiments were performed.

Two fermentation tubes filled with 2 % glucose-broth were inoculated

with mixtures of organisms as below and incubated at 37° C. for 3 days. Result :—

Tube No. 1	Percentage of gas	$\frac{H}{CO_2}$	Voges and Proskauer's reaction
B. cloacae (Jordan) *B. capsulatus* (Pfeiffer) *B. lactis aerogenes* (Escherich) *B. coli communis* (Escherich) *B. acidi lactici* (Hüppe) *B. neapolitanus* (Emmerich) *B. pneumoniae* (Friedländer) *B. proteus vulgaris*	50	$\frac{1}{2}$	+
Tube No. 2			
B. coli communis *B. acidi lactici* *B. neapolitanus* *B. pneumoniae* *B. proteus vulgaris*	20	$\frac{2}{1}$	–

Again fermentation tubes containing as before 2 % glucose-broth were inoculated as below, and after 48 hours' incubation at 37° C. yielded as results :—

	$\frac{H}{CO_2}$	Voges and Proskauer's reaction
No. 1 *B. capsulatus* *B. coli communis* *B. acidi lactici* *B. neapolitanus* *B. pneumoniae* (Friedländer) *B. proteus vulgaris*	11/10	? trace after standing 24 hours, + after 48 hours.
No. 2 *B. lactis aerogenes* *B. coli communis* etc., etc.	1/1	+ after 24 hours.
No. 3 *B. cloacae* *B. coli communis* etc., etc.	6/5	? trace after standing 24 hours, + after 48 hours.
No. 4 *B. capsulatus* (Pfeiffer) *B. proteus vulgaris*	2/3	? trace after standing 24 hours, + after 48 hours.
No. 5 *B. cloacae* *B. proteus vulgaris*	1/2	+ after 24 hours.

A fresh test was made by inoculating fermentation tubes containing bile-salt cane-sugar broth with rabbit faeces + *B. lactis aerogenes*, rabbit faeces + *B. cloacae*, guinea-pig faeces + *B. lactis aerogenes* and guinea-pig faeces + *B. cloacae*. After 48 hours' incubation at 37° C., the results were :—

	Gas %	$\frac{H}{CO_2}$	Voges and Proskauer's reaction
Rabbit faeces + *B. lactis aerogenes*	40	1/1	+
Rabbit faeces + *B. cloacae*	40	1/1	+
Guinea-pig faeces + *B. lactis aerogenes*	10	1/1	trace
Guinea-pig faeces + *B. cloacae*	15	2/1	+

Another series of glucose-broth tubes was inoculated with small quantities of the faeces of various animals and placed at 37° C. After 3 days' incubation the results of growth were :—

Kind of faeces	Percentage of gas in closed arm of tube	$\frac{H}{CO_2}$	Voges and Proskauer's reaction after 24 hours' standing
Rat	80	1/13	–
Donkey	90	3/1	–
Monkey	30	1/5	trace
Rabbit	50	3/1	trace ; increased after 48 hours
Mouse	5	1/0	–
Guinea-pig	8	1/0	–
Goat	1 bubble	1/0	–
Horse	3	1/0	–
Calf	nil		? trace

A similar experiment was made with a broth containing 1 % of cane-sugar instead of glucose. After 5 days' incubation at 37° C. the results were :—

Dog	bubble	1/0	–
Goat	10	3/2	–
Mouse	45	2/3	–
Calf	bubble	1/0	–
Rabbit	30	1/3	–
Monkey	10	2/1	–
Guinea-pig	80	6/1	–
Rat	75	2/3	+
Horse	12	9/1	–

From these results we must conclude that though this test succeeds in artificial mixtures, yet when used with natural mixtures the results are not sufficiently definite to allow us to place absolute reliance upon it as evidence of the presence or absence of the organisms under consideration.

Summary.

(1) The fermentation reactions of certain known organisms have been examined and the lactose-fermenters have been arbitrarily divided into four groups according as they do, or do not, decompose cane-sugar and dulcite, and this grouping has been applied to the organisms isolated in the experiments.

(2) Of human faeces 23 samples have been examined and 241 lactose-fermenting bacilli isolated; of animal faeces 25 samples giving 239 organisms; of milk 4 samples and 40 organisms; and of a mixture of tap-water and faeces 7 samples and 75 bacteria.

(3) Of 480 *coli*-like organisms fermenting lactose isolated from human and animal faeces:—

120 (25 p.c.)	belonged to the	1st group	represented by the		*B. acidi lactici* (Hüppe);
178 (37 p.c.)	„	2nd	„	„	*B. coli communis* (Escherich);
110 (23 p.c.)	„	3rd	„	„	*B. neapolitanus* (Emmerich);
72 (15 p.c.)	„	4th	which contains organisms fermenting cane-sugar but not dulcite;		

or, to put it more succinctly 298 (62 p.c.) had no action on cane-sugar, and 182 (38 p.c.) fermented this sugar, and 288 (60 p.c.) decomposed dulcite, while 192 (40 p.c.) did not.

(4) The *B. acidi lactici* (Hüppe) group in certain circumstances disappeared entirely from the faeces.

(5) In all 625 lactose-fermenting bacilli have been isolated, and of these only 4 have been identified as of the *B. lactis aerogenes* type and 19 as *B. cloacae.*

(6) 18 *B. cloacae* were planted on gelatine and examined from time to time during six weeks when they did not show a trace of liquefaction but there was distinct liquefaction in three months.

(7) The *B. coli communis* (Escherich) has been exposed for 358 days to the influence of unfavourable environment without losing any of its fermentative characteristics, or its power of clotting milk and producing indol.

(8) Voges and Proskauer's "Kalilauge-roth-reaktion" has been looked for in the cultures of a large number of organisms and has only appeared when organisms of the *B. lactis aerogenes* or *B. cloacae* groups were present.

(9) At present there is no means of differentiating the lactose-fermenting organisms of human from those of animal origin; or those of normal dejecta from those found in enteritis.

(10) *B. lactis aerogenes* is not the same organism as the *B. acidi lactici* (Hüppe), and these organisms are not simply non-motile forms of *B. coli communis.*

(11) Contrary to Péré's statement, there are dulcite-fermenting *coli* in the faeces of adults as well as of sucklings.

(12) As the *B. coli communis* group seems to be more clearly associated with faeces than the other groups, further research along these lines would in all probability help to place the value of "*coli*" as an index of pollution upon a sound basis.

(13) As the *B. acidi lactici* (Hüppe) group sometimes disappears so quickly from the faeces, it may not be of truly faecal origin, or may require such special conditions for its continued growth that it may eventually come to afford evidence as to the nearness or remoteness of pollution.

(14) In the report of the bacteriological examination of a drinking water all the characters of the organisms isolated should be given, and these should include at least the fermentation reactions in glucose, lactose, mannite, cane-sugar, and dulcite, the gas amount and gas-ratio, and the occurrence of Voges and Proskauer's reaction.

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OBSERVATIONS UPON FIBRIN-FERMENTS IN THE VENOMS OF SNAKES AND THE TIME-RELATIONS OF THEIR ACTION.

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(One Figure in text.)

In 1893 [1] I found that ·0001 grm. per kilo or upwards of the venoms of two Australian snakes, *Pseudechis porphyriacus* and *Notechis scutatus*, when introduced into the blood stream of dogs, cats and rabbits caused intra-vascular clotting. This action also occurs when the snake discharges a relatively large amount of venom into small animals, as when seizing frogs or mice as prey, but is not a usual phenomenon of poisoning by these snakes in man, for reasons which I have discussed elsewhere [2].

On further study I found that still smaller quantities produced a transient increase followed by diminished coagulability and in some cases prevented clotting indefinitely. In this and in some other respects the effects of venom upon coagulation were so closely analogous to those discovered by Wooldridge to follow nucleo-proteid injections, that I was at the time inclined to interpret the phenomena I was studying as really occasioned by the setting free in some way of such substances.

Some years later (1903) Lamb [3] pointed out that the addition of the venom of *Vipera russellii* to citrate plasma produced clotting *in vitro*. These experiments of Lamb indicated a much simpler explanation of the phenomenon, and led me to the conclusion that my previous interpretation was erroneous.

Subsequently Lamb [4] found the venom of *Echis carinatus*, another Indian viperine snake, to be much more effective than Daboia poison in producing clotting of citrate plasma and that it also clotted oxalate plasma. On trying the effect upon oxalate plasma of the venoms of *Notechis scutatus* and *Pseudechis porphyriacus* and of the poisons of *Echis carinatus* and of *Vipera russellii*, kindly sent me by Capt. Lamb, I found that the power of the three first greatly exceeded that of *Vipera*

russellii. The small quantity of *Notechis* venom necessary to produce clotting is very striking, as is instanced by the following experiments:—

To 2 c.c. oxalate plasma (dog) was added 1 drop = ·017 c.c. of solution of venom of the following strengths, and the mixture placed in a bath at 40° C.

·1 % =	·00017 grm.	Clotted in less than 1 minute.
·01 % =	·000017 ,,	Clotted in less than 1·5 minutes.
·001 % =	·0000017 ,,	Clotted in less than 15 minutes.
·0001 % =	·00000017 ,,	Clotted in less than 10 hours.
·00001 %		Permanently fluid.
Control		Permanently fluid.

As a considerable part of the solid matter in venom consists of substances which have not this action, the small quantity required is certainly surprising.

The venom of another colubrine snake, *Pseudechis porphyriacus* (the Australian black snake), is nearly equally active and a sample of the venom of *Echis carinatus* tested in the same way possessed from $\frac{1}{2}$ to $\frac{2}{3}$ the potency. The venoms of many viperine snakes contain substances possessed of similar powers in varying degree, for Noc[5] has observed the same action with the venoms of four species of *Lachesis.*

These venoms appear to contain veritable fibrin ferments and their clotting power is not due to their action upon prothrombins or thrombogens contained in the plasma as the presence of calcium ions is unnecessary. They clot 10 % oxalate plasma as rapidly as ·2 % and the action is the same upon oxalate, citrate, fluoride and magnesium sulphate plasma, and also upon hydrocele fluid and solutions of fibrinogen prepared from the horse by Hammarsten's process.

Properties of the ferment Solution. The ferment contained in the venoms does not appear to be used up in the process. If 5 c.c. of plasma be clotted with one drop of ·1 % solution of venom and the clot removed, and to the serum another 5 c.c. of plasma be added, this is clotted. This can be repeated four or five times, coagulation occurring more and more slowly with each successive addition. The experiment cannot be repeated indefinitely, nor so many times as would be expected from the calculated minimal clotting dose, as presumably each successive crop of fibrin carries away adsorbed ferment.

Heating ·1 % solution of venom to 75°C. for 10—15 minutes completely destroys this ferment action and, as found by Lamb[6], the destruction occurs the more quickly the weaker the solution. It is also slowly destroyed in water at ordinary temperature and more rapidly the greater the dilution.

The ferment dialyses slightly, and when a ·01 % solution of venom is filtered through 8 % gelatine[7] the filtrate possesses about $\frac{1}{200}$th of the ferment-power of the original solution.

Specificity. The clotting ferments contained in different venoms are not identical. The serum of a horse immunised with the poison of *Notechis scutatus* contains an anti-ferment which neutralises the ferment in this venom. Thus 0·1 c.c. of a serum, for which I am indebted to my friends Dr Ashburton Thompson and Dr Tidswell of the Board of Health, N. S. Wales, proved, when tested against the corresponding venom, to be capable of neutralising upwards of 10,000 minimal clotting doses for 1 c.c. of plasma.

It was equally effective against the ferment in the poison of *Pseudechis porphyriacus,* another Australian colubrine snake, but 200 times the quantity was without effect upon the ferment of *Echis carinatus,* an Indian viperine snake.

It had no neutralising action against Schmidt's or Hammarsten's fibrin ferment solutions, nor does it influence in the least the clotting of oxalate plasma on the addition of calcium ions.

The oxalate plasma of an animal immunised with the venom of *Notechis* requires, as might be expected, considerably greater quantities of the ferment (200—300 times) to produce clotting.

This specificity is not surprising in view of the multiplicity of fibrin ferments shown to exist by Bordet and Gengou[8] and Morawitz[9].

Time relations of the ferment action. Effect of temperature on the rate of action.

I have determined the effect of temperature on the coagulation rate, between the temperatures 6·3° C. and 40° C. The observations were made with ·4 % fluoride plasma and ·01 % solution of the venom of *Notechis.* The temperature during the experiments was kept constant by inserting all the solutions in a water bath.

The following are the values obtained:

Temperature	40°	Time	60″, 61	60·5	Temperature	10°	Time 214″, 220	217
,,	30°	,,	65, 64·8	65	,,	7·5°	,, 300, 310	305
,,	20°	,,	95, 94	94·5	,,	6·3°	,, 343, 348	345
,,	15°	,,	124, 126	125				

These are expressed graphically in Fig. 1; here it is seen that the fall in velocity between 40° and 30° C. is small, between 30° and 15° C. con-

siderably greater, and that between 15° and 6° C. the temperature co-efficient increases very rapidly.

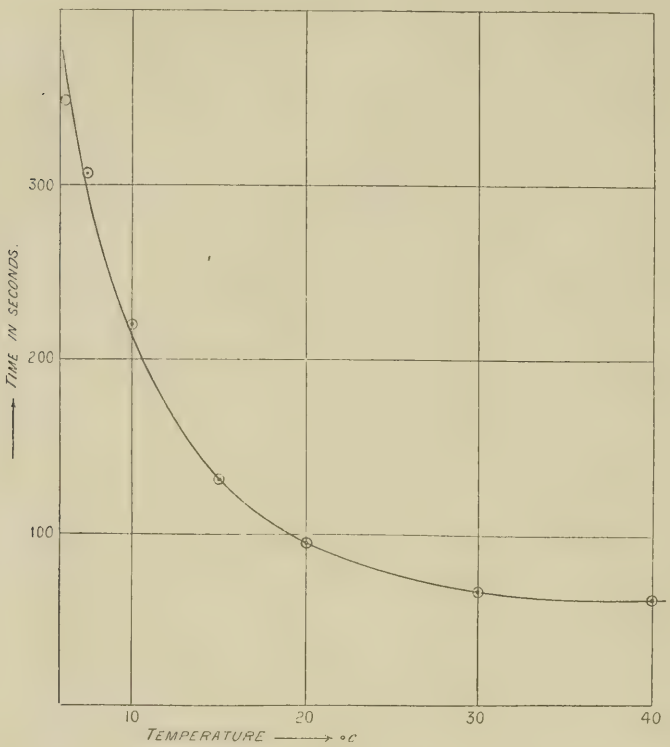

Fig. 1. ·017 c.c. of ·01 % sol. *Notechis* Venom = ferment sol. Effect of temperature on fibrin ferment action. Fluor plasma dog.

Effect of quantity of ferment upon rate of action. The influence of quantity of ferment upon the time of coagulation has been studied by Fuld[10] and Fuld and Spiro[11]. Fuld used turkey or goose plasma prepared by Delezenne's[12] method and an extract of muscle in ·8 % NaCl solution as ferment.

Fuld found, in a particular instance, that doubling the quantity of ferment solution increased the velocity one and a half times and expressed this fact by the following equation :—

$$\log y_0 - 0{\cdot}585 \log x = \log y$$

$$\left({\cdot}585 = \frac{\log 1{\cdot}5}{\log 2}\right),$$

where x equals quantity of ferment which will cause clotting in

time y and x_0 quantity in time y_0; x_0 being taken as the unit quantity for purposes of calculation disappears.

On employing this formula to calculate the clotting times in a series of experiments Fuld found an extremely good agreement between calculated and observed results. In his later experiments Fuld[13] evidently found that the ratio $\frac{1 \cdot 5}{2}$ does not always represent the relation of velocity increase to ferment increase, so that ·585 in the above equation should be replaced by a constant, the value of which must be determined for each plasma.

My own experiments were made in a very simple manner. I used oxalate plasma of the sheep and dog, obtained by drawing blood into an equal quantity of ·8 % NaCl solution containing ·4 % of oxalate of potassium and centrifuging off the corpuscles. In every case the volume of fluid was identical and amounted to 1·17 c.c., *i.e.* 1 c.c. of plasma and 10 drops of mixed venom and saline from a particular drop-pipette. The venom solution and saline were carefully placed in the bottom of a dry test-tube without wetting the sides. To this was added the 1 c.c. of plasma and at the moment the plasma had been delivered a stop watch was released. The whole of the tubes and fluids were kept at a temperature of 40° C. before and during the experiment by means of a water bath.

One had to decide upon an arbitrary standard for an end point, and I took for this purpose the moment when the tube could be inclined without the clotting plasma following the deflection of the tube. Some warning of the moment when solidification was about to occur was afforded by a sudden turbidity of the fluid. All the experiments were done in duplicate.

When the times for clotting with varying amounts of ferment were considered, it was obvious that, with the exception of the experiments made with the larger quantities of ferment solution, the relationship was a simple one and that time multiplied by quantity of ferment equalled a constant.

In the three series of experiments in Tables 1, 2 and 3 with two different venoms and two sets of plasma, the agreement between observed and calculated values is a close one. The deviation from this law is, however, marked as soon as the time of clotting is reduced to about 100 seconds, and with 200 fold the amount of ferment added, clotting could not be induced in less than 22 seconds.

I have attempted to determine the time law for these higher

quantities of ferment, but with short times the errors of experiment were too great to draw quantitative conclusions from the observations. Irregularity in the time of clotting was also noticed when the interval was greater than 20 minutes, *i.e.* with very small quantities of ferment. Under these circumstances successive clots also occurred which made it impossible to make accurate observations.

The difference between the results of Fuld and myself is not, I think, difficult to explain. For reasons mentioned above, I regard the ferments contained in these venoms as catalysts inducing the change in the fibrinogen. This is not the case with the tissue extracts employed by Fuld, for A. Morawitz[14] has shown that such extracts do not clot fibrinogen solutions nor oxalate plasmas and their action is an indirect one, viz. to convert a thrombogen present in the turkey or goose plasma prepared by Delezenne's method into prothrombin, which in the presence of calcium ions is converted into a fibrin ferment; in other words the tissue extracts contain a kinase.

Exp. 1. *Oxalate Plasma of Dog,* 1 *c.c. and* ·005 % *solution of the venom of Notechis scutatus.*

Total volume = 1·17 c.c. Temperature 40° C. 1 drop = ·0172 c.c.

Number of drops added	Observed times of clotting, in seconds	Mean of observed times	Calculated time of clotting
10	70 72	71	31
5	85 82	83	62
4	97 96	96	78
3	102 110	106	104
2·5	130 130	130	126
2	142 156	150	156
1·5	185 190	188	208
1·1	295 270	282	284
1	315 310	312	312
·5	650 610	630	624

Exp. 2. *Oxalate Plasma of Dog,* 1 *c.c. and* ·005 % *solution of the venom of Echis carinatus.*

Total volume = 1·17 c.c. One drop = ·0172 c.c. Temperature 40° C.

Number of drops added	Observed times of clotting, in seconds	Mean of observed times	Calculated time of clotting
10	62 60	61	48
5	95 101	98	96
4	128 120	124	120
3	159 151	155	161
2	245 237	241	241
1	507 495	501	482

Exp. 3. *Oxalate Plasma of Sheep,* 1 *c.c. and* ·005 % *solution of the venom of Notechis scutatus.*

Total volume = 1·17 c.c. One drop = ·0172 c.c. Temperature 40° C.

Number of drops added	Observed times of clotting, in seconds	Mean of observed times	Calculated time of clotting
10	72 72	72	44
5	103 110	106	88
4	114 117	115	110
3	139 135	137	147
2·5	164 180	172	177
2	227 215	221	221
1·5	287 298	292	295
1	445 450	447	442
·5	860 881	870	884

In their later work Fuld and Spiro[15] interpret the action of tissue extracts upon Delezenne's plasma and also upon peptone plasma in the same manner. The fact that the constant in Fuld's equation needs changing for each plasma further supports this view.

Under these circumstances the rate of coagulation would not depend only upon the amount of kinase present and could not be expected to follow the same simple law.

Influence of concentration of fibrinogen in the solution upon the velocity of reaction. The concentration of the fibrinogen unlike that of the ferment exerts only a very small effect upon the velocity of the reaction. In the experiment recorded below some oxalate plasma was successively diluted with an equal bulk of saline so as to represent concentrations of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$. These were added in equal volume to the same quantity of ferment. All operations were conducted at 40° C.

Experiment showing influence of concentration of fibrinogen on reaction-rate.

Concentration of plasma	Time of clotting		
	Obs. 1	Obs. 2	Mean
1	54″	57″	55·5″
$\frac{1}{2}$	55	57	56
$\frac{1}{4}$	65	62	63·5
$\frac{1}{8}$	72	70	71
$\frac{1}{16}$	90	82	86

Even the diminution in velocity indicated above is exaggerated, because with the weaker solutions of fibrinogen a proportionately larger quantity must be converted to fibrin before the end point decided upon is reached.

The same law of inverse proportionality between time and quantity of ferment has been found to hold for the clotting of caseinogen by rennet by Segelcke and Storch[16], Hansen[17], Soxhlet[18], and Fuld[19]. Fuld has worked at the time law in this case very thoroughly and found, as was previously pointed out by Duclaux[20], that the law of inverse proportionality only held for moderate quantities of rennet, and was departed from in the case of quantities which caused clotting in less than two minutes and also with very small quantities of ferment; much as has been found to be the case in the experiments with fibrin ferments recorded above. A similar inverse proportionality for intermediate concentration of ferments has been observed by O'Sullivan and Thompson[21] with invertase, Vernon[22] for peptone splitting ferments, and Bayliss[23] and Hedin[24] for trypsin.

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SOME OBSERVATIONS UPON THE MICRO-ORGANISMS OF MEAT POISONING AND THEIR ALLIES.

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THE typical representative of the bacterial flora of the normal intestine is the colon bacillus, its natural habitat being the digestive tract of man and animals. The typhoid bacillus is the most familiar example of those organisms which from time to time invade the intestine of man and produce disease. The colon and the typhoid bacillus, besides this contrast in infective properties, present biological and cultural features by means of which they can be differentiated outside the body. In addition to these established types, there occur in the healthy, and diseased, intestine a large number of organisms of an intermediate character, some approaching to the colon, others to the typhoid type. The colon group, for example, has been found to embrace a number of varieties of undoubtedly the same species. These varieties do not, however, appear to possess any special pathological significance. On the other hand, there occur amongst the intermediate forms, species of micro-organisms with distinct pathogenic properties for man and animals, of which the dysentery, the hog cholera, the meat poisoning and the paratyphoid group have now been identified. It has further been shown that relationships exist between certain of these groups of organisms, as evidenced by the possession of common biological properties. This is particularly the case with regard to the agents of various septicaemias in the lower animals, notably the hog cholera group, and the affinity its members bear to the meat poisoning, and paratyphoid, group of bacteria.

The life-history of such of these organisms as are pathogenic for man and their possible derivation from animal sources—cattle and swine—is a subject that merits careful and prolonged investigation. The problems raised are akin to those met with in other fields of research on the intercommunicability of disease agents—for example, bovine and human tuberculosis, rat plague, and human plague. The refinement of bacteriological methods has enabled a con-

siderable advance to be made in the classification of the septicaemic organisms, whilst the agglutination test has furnished a delicate method of sifting out the homologous species of such pathogenic bacteria. At the same time but little definite knowledge exists as to the distribution of these organisms outside the actual morbid process.

The main object of the present research was to investigate the distribution of organisms of the meat poisoning and paratyphoid groups, more particularly in the intestines of healthy animals. I have endeavoured to determine in how far the organisms isolated from the normal intestine conformed in type to those met with in certain septicaemias of animals, and in meat poisoning, and paratyphoid infections in man.

Historical.

Meat poisonings are those diseases in man, usually epidemic in character, due to the consumption of meat from unhealthy animals—generally cattle (cow, calf), and more rarely the horse, pig, and goat. The animals commonly suffered from enteritic or septic processes. The agents of these diseases in animals produce at times in man a septic or infective enteritis.

Ostertag[1] records from 1880-1903 ninety outbreaks of this form of meat poisoning, with about 4,000 cases. One may assume that the number of unrecorded cases was also considerable.

The symptoms vary in character and severity, and three forms of the disease have been described, namely, a gastro-enteritic, a choleraic, and a typhoidal.

An important fact to be noted is that such cases of poisoning are not due to putrid meat or its toxins. The meat appears to be healthy, and is without smell or unpleasant taste. The poisoning is due to virulent bacteria present in the meat which invade the intestines and body.

One must differentiate from this another form of food poisoning due to a *post-mortem* multiplication of saprophytic bacteria in a foodstuff, resulting in the production of highly toxic substances. The latter is usually termed allantiasis, botulism, or sausage poisoning. The disease is caused by eating decomposed sausages, or other forms of preserved meat (hams, etc.). The attack is characterized by nervous symptoms of central origin, disturbances of secretion, motor disturbances, mydriasis, ptosis, etc. The researches of van Ermengem[2] have shown that this disease is due to the toxins of an anaërobic saprophyte—the bacillus botulinus. The meat comes from apparently healthy animals, and acquires its poisonous properties subsequently. The symptoms are due not to an infection, but to an intoxication with a poison produced by the B. botulinus in the meat consumed. In botulism the nervous symptoms predominate, whilst in true meat poisoning the gastro-intestinal symptoms constitute the main clinical feature. The infective organisms in meat poisoning also produce a toxin, as cases are recorded after the consumption of cooked meat or of soup made from it.

The first exact bacteriological investigation of meat poisoning was made by Gaertner[3] during an outbreak at Frankenhausen in 1888. Fifty-eight individuals became ill after eating the meat from a cow which had sickened and been slaughtered. The symptoms were those of an acute gastro-enteritis, with severe general symptoms. Gaertner found in the muscles

and spleen of a fatal case a bacillus and also large numbers of the same organism in portions of the meat. This organism he termed the B. enteritidis. Its cultures were fatal to mice on feeding, and also on inoculation into guinea-pigs and rabbits. The necropsy showed acute enteritis, haemorrhages in the serous cavities, lungs and kidneys, and pneumonia. The killed cultures were likewise fatal to experimental animals, and contained a toxin. This toxin, it is stated, may be heated to boiling point without destruction. The B. enteritidis thus differs from the colon organism in its marked virulence and in the production of heat-resistant toxins.

Organisms identical with, or closely allied to, the B. enteritidis have been isolated by a number of observers in subsequent outbreaks of meat poisoning. Of these a very full account will be found in a paper by Professor Bernhard Fischer, Zur etiologie der Sogenannten Fleischvergiftungen, published in the *Zeitschrift für Hygiene*, vol. xxxix, p. 447, 1902.

There is general agreement as to the main characteristics of the meat-poisoning organisms of the B. enteritidis type. They are motile short rods, non-sporing, non-liquefying, and decolorized by Gram's method. They differ from typhoid in their colon-like growth on potato and their fermentation of grape sugar, and, on the other hand, from colon in their fermentation of grape sugar, but not of cane and milk sugar. The organisms do not curdle milk, and the majority produce no indol. They further differ from colon and typhoid in their marked infectivity for experimental animals and the property they possess of forming heat-resistant toxins. The B. enteritidis is infective per os and penetrates into the blood and organs, producing a haemorrhagic septicaemia.

The infectivity of the meat-poison organisms and their biological characters point to an affinity with the hog-cholera group of bacteria. I have obtained cultures of many of these organisms and controlled their biological and cultural characteristics.

The study of the meat-poisoning and hog-cholera group has received added importance through the discovery of the paratyphoid infections in man—that is, those forms of disease which, although presenting the clinical symptoms of a mild attack of enteric fever, are due not to an infection with the typhoid bacillus, but with organisms termed "paracolon" or "paratyphoid." These organisms have been isolated directly from many of the cases recorded, and the blood of the patients gives a marked agglutination with their cultures, but not with those of the B. typhosus. Attention was first drawn to the paracolon or paratyphoid bacilli by Achard and Bensaude.[4] These organisms have been particularly studied in Germany by Schottmüller, Brion and Kayser, and others, and in America by Gwyn, Cushing, Buxton, and Coleman, etc. A good summary of the literature is given by Libman in the *Journal of Medical Research*.[5] These organisms likewise present affinities to the Gaertner and hog-cholera group, and suggest a possible interrelationship of paratyphoid with analogous animal diseases. The mouse typhoid bacillus of Loeffler is another organism that presents similar relationships, and also the B. psittacosis. Psittacosis is a contagious disease of parrots, communicable to man, marked by pulmonary disorder and high fever. The organism is fully described by Nocard and Leclainche.[6]

We have, therefore, in meat poisoning, and in psittacosis, diseases derived from animal sources. It remains to be determined in how far the paratyhoid infections have their origin in analogous diseases of the lower animals.

Author's Experiments.

Morphologically, the bacilli of the enteritidis groups are practically indistinguishable from B. coli; they are actively motile, and do not retain the stain by Gram's method.

Their cultural peculiarities were found to be as follows: When grown on peptone beef broth and incubated for twenty-four hours at 37° C., universal turbidity of the broth is produced, with or without the formation of a pellicle. If grown on this medium for five days at 37° C., no indol—or the merest trace—is found to be present. If, however, this five days' old broth be filtered through a Pasteur-Chamberland filter a toxic filtrate is obtained, as can be readily proved by injecting 2 c.cm. intraperitoneally into a guinea-pig, the animal dying within twenty-four hours. Van Ermengem (loc. cit.) states that in the case of the meat poison bacillus he isolated in the Moorsele epidemic, this toxin did not lose its virulence when heated to 100° C., or even 120° C. The growth on agar and gelatine is creamy and moist, resembling that of the bacillus coli, there being no liquefaction of the gelatine. When grown on the various sugar media at 37° C., this group of organisms will be found to produce acid and gas on dulcite, mannite, and glucose broths, but not to affect lactose or cane-sugar broths. The most characteristic reaction, however, is that on litmus milk, which, incubated at 37° C., becomes acid in twenty-four hours, and then, after a few days, strongly alkaline. No clotting occurs even at the end of a month. The period at which this alkaline reaction takes place varies with the different members of the group, the B. enteritidis of Gaertner, for instance, generally completing the reaction within forty-eight hours, whilst the B. paratyphoid B of Schottmüller may take from eight to ten days.

The reactions on Petruschky's litmus whey are similar to those on litmus milk, except that the change from acid to alkaline is more rapid in the former medium, taking place in some members of the group at the end of twelve hours.

It will be noticed from the table of cultural reactions that the enteritidis group, with a few notable exceptions, includes most of the food-poisoning bacilli. It includes also B. typhi murium, B. psittacosis, B. hog cholera of Evans, B. hog cholera "Maryland" of Theobald Smith, and the B. paratyphoid B of Schottmüller.[7] This latter differs slightly from the rest of the group, producing marked indol when grown in broth at 37° C. for five days.

Some of the organisms under consideration, though nearly allied to the enteritidis group, do not conform to them in their cultural reactions. The B. paratyphoid A of Schottmüller, for instance, and the B. paratyphoid of Brion and Kayser[8] have both been isolated from cases clinically resembling typhoid fever. These two bacilli are culturally indistinguishable from each other, and differ from the rest of the enteritidis group in that they produce indol when grown on broth for five days, and when grown on litmus milk the acid reaction is not followed by any alkaline reaction, even at the end of a month, the milk remaining acid without clotting.

The other bacilli, from those under consideration, whose cultural reactions do not conform to those of the enteritidis group, are the food-poisoning bacilli of Wesenberg, Grünthal, and Friedeberg,[9] and the hog-cholera bacilli of McFadyean and the "Arkansas" of Theobald Smith.

The B. Wesenberg has practically no resemblance to the members of the enteritidis group; it produces acid on all the sugars, producing gas, however, only on glucose and cane sugar. Litmus milk is rendered acid without clotting; no alkaline reaction takes place, and, when grown on broth, indol is produced at the end of five days.

The B. Grünthal resembles the members of the colon group in cultural reactions, being, except for its motility indistinguishable from B. acidi lactici of Hüppe. It produces acid and gas on glucose, mannite, and lactose, broth, acid and clot on litmus milk, and indol on beef broth when grown for five days at 37° C.

The B. Friedeberg differs from the rest of the series in producing no change on any of the before-mentioned sugars. excepting cane sugar, on which it produces acid, but no gas, It creates general turbidity on peptone broth at 37° C. in twenty-four hours, but no indol at the end of five days.

Thehog-cholera bacillus of McFadyean differs widely in cultural reactions from the rest of the enteritidis group, as well as from the other hog-cholera bacilli. It produces acid, but very slight gas on glucose broth, no reaction on mannite, or dulcit broth, acid without clotting, and no alkaline change on litmus milk, and slight indol on broth at the end of five days; in fact, its sole resemblance to the enteritidis group consists in its producing no change on lactose or cane-sugar broth, and in its being a motile bacillus which does not stain by Gram's method.

The B. hog cholera "Arkansas" of Theobald Smith differs from the enteritidis group in producing no change on dulcit broth when grown at 37° C. for fourteen days, in which respect it differs also from his "Maryland" culture, as well as by its production of a scum on peptone beef broth at 37° C. at the end of twenty-four hours. In other respects the hog cholera "Arkansas" resembles the rest of the enteritidis group. It produces acid and gas on glucose, and mannite, broths, no change on lactose, or cane sugar broth, acid and subsequently alkaline on litmus milk, and no indol on peptone beef broth at the end of five days.

To recapitulate, therefore: As far as morphological and cultural reactions are concerned, the following bacilli fall into the enteritidis group: The food poisoning bacilli of Gaertner, Aertrycke, Moorsele, Hanstedt, Breslaviensis, B. morbificans bovis, Günther, Abel, and Rennfleth; also B. typhi murium, B. psittacosis, B. hog cholera "Maryland" of Theobald Smith, and B. hog-cholera of Evans.

The remaining bacilli that have been experimented with for the purposes of this research differ culturally from the above, and also from each other, excepting B. paratyphoid A of Schottmüller and B. paratyphoid of Brion and Kayser, which are culturally identical.

Having determined, therefore, that, as regards cultural and biological characteristics, the bacilli of the enteritidis group and of the Paratyphoid A group of Schottmüller include most of the organisms that give rise to food poisoning, and to cases of disease resembling typhoid fever, I proceeded to investigate

the question as to whether the bacilli of these two groups were represented by analogous types in the intestines of normal animals.

To this end the following intestinal material was submitted to investigation: The faeces of guinea-pigs, rabbits, sheep, and pigs obtained from a large number of the various animals; also the scrapings from the mucous surfaces of the large and small intestines of three pigs, three sheep, two bullocks, one horse, one calf, and of a child that died in Guy's Hospital from bronchopneumonia.

The method adopted was as follows: An emulsion of the substance under examination was made with distilled water, and about ½ c.cm. of the emulsion was added to tubes of bile salt dulcit broth coloured with neutral-red (MacConkey), and incubated for twenty-four hours at 42° C. Of this culture 4 c.cm. was then injected subcutaneously into a number of guinea-pigs. The guinea-pigs that died within four days were opened, aseptic precautions being taken, and the heart exposed and seared with a hot iron. The heart blood was pipetted with a sterile pipette into tubes of bile-salt dulcit broth, and incubated at 42° C. for twenty-four hours. If at the expiration of this time acid and gas were produced, neutral-red bile-salt agar plates were inoculated and incubated at 42° C. for twenty-four hours. The colourless colonies were then picked off (the bacilli of the two groups for which the search was made producing no acid on lactose, their colonies are colourless), and from them bile-salt dulcit-broth tubes were inoculated, and incubated at 42° C. for twenty-four hours. The tubes that gave the acid-and-gas reaction were then used to inoculate the following media: Glucose broth, dulcit broth, mannite broth, lactose broth, cane-sugar broth, peptone-beef broth, litmus milk, agar, and gelatin, in order to determine in how far the cultural reactions of the organisms isolated agreed with those of the B. enteritidis and Paratyphoid A groups. All the tubes were incubated at 42° C., excepting the gelatine, which was incubated at 20° C.

It will be noted that only those guinea-pigs were tested that died within four days after injection, those dying from a more chronic infection being rejected. This naturally considerably diminished my positive results, but at the same time excluded any possibility of error which might arise through an accidental infection from the sick to the healthy animals, or other causes, though all precautions against such infection were of course taken. The positive results were, however, fairly numerous, and were as follows:

Twenty-one cultures of bacilli, of the enteritidis type, and answering to the characteristic morphological and cultural tests were isolated, namely, motile bacilli of the size and appearance of the B. enteritidis of Gaertner, not staining by Gram's method, producing a cream-coloured growth on agar and gelatine, and not liquefying the latter medium, giving acid and gas on glucose, dulcit and mannite broths, but not affecting lactose, or cane sugar broths, and rendering litmus milk at first acid and then alkaline, without clotting.

Also ten cultures of the Paratyphoid A type were isolated, which agreed culturally and morphologically with the former, excepting in their reaction on litmus milk, which remained acid without clotting, and in the production of indol on broth at the end of five days.

Cultural Reactions of known

Bacterium.	Morph	Broth.	Glucose.	Mannite	Lacto
B. enteritidis, Gaertner, Original A.	B Motile	G. T.	A. G.	A. G.	Nil
B. enteritidis, Gaertner, Original B.	B. Non-Motile	,,	,,	,,	,,
B. Gaertner, L.I.P M. ...	B. Motile	,,	,,	,,	,,
B. Gaertner, "Guy's" ...	,,	,,	,,	,,	,,
B. Gaertner, "Van Ermengem"	,,	,,	,,	,,	,,
B. typhi murium	,,	,,	,,	,,	,,
B. Moorsele	,,	,,	,,	,,	,,
B. psittacosis, Nocard ...	,,	,,	,,	,,	,,
B. Haustedt, "Fischer"...	,,	,,	,,	,,	,,
Food poisoning, Abel ...	,,	G. T S.	,,	,,	,,
B. Rennfleth, "Fischer"...	,,	G. T.	,,	,,	,,
B. icteroides, Sanarelli ...	,,	G. T S	,,	,,	,,
B. epidem. jaundice ...	,,	,,	,,	Nil.	,,
B. Aertrycke, "Van Ermengem"	,,	G. T	,,	A. G	,,
B. hog cholera, Evans ...	,,	,,	,,	,,	,,
B. hog cholera, Maryland, Theobald Smith	,,	,,	,,	,,	,,
B. hog cholera, Arkansas, Theobald Smith	,,	G. T. S.	,,	,,	,,
B. hog cholera, McFadyean	,,	G. T.	A. G. Slt.	Nil	,,
B. Breslaviensis	,,	,,	A. G.	A. G	,,
Food poisoning, Gunther	,,	,,	,,	,,	,,
B. paracolon, "Day" ...	,,	G. T. S.	,,	,,	,,
B. paratyphoid A, Schottmüller	,,	G. T.	,,	,,	,,
B. paratyphoid B, Schottmüller	,,	,,	,,	,,	,,
B. paratyphoid, Brion and Kayser	,,	,,	,,	,,	,,
B. morbificans bovis ...	,,	G T. S.	,,	,,	,
B. Wesenberg	,,	G. T.	,,	A.	A
B. pseudo-tuberculosis, "Pfeiffer"	B. Non-motile	,,	A	,,	N
B. Friedeberg, Gaffky and Paak	,,	,,	Nil	Nil	,
B. Grunthal	B. Motile	,,	A. G.	A. G.	A.
B. coli, Escherich	,,	,,	,,	,,	,
B. acidi lactici, "Hüppe"	B. Non-motile	,,	,,	,,	,
B. lact. aërogenes ... [key	,,	G. T. S.	,,	,,	.
B. coli, T. W. F. 6 MacConkey	B. Motile	G T.	,,	,,	.
B. coli, T. W. F. 13 MacConkey	B. Non-motile	,,	,,	,,	.
B. cloacae	B Motile	,,	,,	,,	
B. pyogenes foetidus ...	B. Non-motile	,,	A.	A.	
B. typhoid, Delépine ...	B. Motile	,,	,,	,,	N
B. typhoid L.I.P.M. ...	,,	,,	,,	,,	
B. dysentery, Shiga ...	B Non-motile	,,	,,	Nil	
B. dysentery, Kruse .	,,	,,	,,	,,	
B. dysentery, Flexner, Philippines	,,	,,	,,	A.	
B. dysentery, Gray ...	,,	,,	,,	,,	

A. = Acid.
Alk. = Alkaline.
G. T. = General turbidity
A. G. = Acid and gas.

Dulcit.	Lit. Milk.	Indol.	Agar.	Gelatine	Litmus Whey.	
				No Liq.	4 hrs.	8 days.
A. G.	A. then Alk.	o	Creamy, raised, moist, translucent	Creamy, raised, moist, translucent	A.	Alk.
,,	,,	o	,,	,,	,,	,,
,,	,,	o	,,	,,	,,	,,
,,	,,	o	,,	,,	,,	,,
,,	,,	o	,,	,,	,,	,,
,,	,,	+ trace	,,	,,		
,,	,,	o	,,	,,		
,,	,,	o	,,	,,		
,,	,,	+ trace	,,	,,		
,,	,,	+ Slt.	,,	,,		
,,	,,	,,	,,	,,		
Nil.	,,	+	,,	,,		
,,	,,	+	,,	,,		
A. G.	,,	Trace	,,	,,	,,	,,
,,	,,	,,	,,	,,		
,,	,,	o	,,	,,		
Nil	,,	o	,,	,,		
,,	A.	Trace	,,	,,	,,	A.
A. G	A. then Alk.	o	,,	,,		
,,	,,	o	,,	,,	,,	Alk.
Nil	,,	o	,,	,,		
A. G.	A.	+	,,	,,	,,	A.
,,	A. then Alk.	+ Slt.	,,	,,	,,	Alk.
,,	A.	Trace	,,	,,	,,	A.
,,	A. then Alk.	+	,,	,,	,,	Alk.
A.	A.	,,	,,	,,	,,	A.
Nil	Nil	,,	,,	,,	Nil	Nil.
,,	,,	o	,,	,,		
,,	A. C.	+	,,	,,		
A. G.	,,	,,	,,	,,		
Nil	,,	,,	,,	,,		
,,	,,	o	,,	,,	A.	A.
A. G.	,,	+	,,	,,		
Nil	,,	+	,,	,,		
,,	,,	o	,,	,,		
A.	,,	+	,,	,,		
Nil	A.	o	,,	,,	,,	,,
,,	,,	o	,,	,,	,,	,,
,,	A. then Slt. Alk.	o	,,	,,	,,	Alk.
,,	,,	o	,,	,,	,,	,,
,,	,,	+	,,	,,	,,	,,
,,	,,	+	,,	,,	,,	,,

A. C. = Acid and clot.
A. then Alk. = Acid then alkaline.
S. = Scum
Slt. = Slight.

A. Cultures Obtained from Faeces.

From rabbits' faeces, 3 cultures of the Paratyphoid A type ;
,, guinea-pigs' faeces, 1 culture of the Paratyphoid A type ;
,, sheep's faeces, 1 culture of the B. enteritidis type ; and
,, pigs' faeces, 1 culture of the B. enteritidis type of organisms.

B. From the Scrapings of the Mucous Surface of Large Intestines.

From 3 pigs, 4 cultures of B. enteritidis, and 1 of the Paratyphoid A type ;
,, 2 bullocks, 4 cultures of the B. enteritidis type ;
,, 3 sheep, 1 culture of the B. enteritidis, and 1 of the Paratyphoid A type ; and
,, 1 calf, 1 culture of the B. enteritidis group.
The results in the case of the child's and horse's intestine were negative.

C. From the Scrapings of the Mucous Surface of Small Intestines.

From 3 pigs, 7 cultures of the B. enteritidis group ;
,, 2 bullocks, nil ;
,, 3 sheep, 1 culture of the Paratyphoid A type ;
,, the calf, 1 culture of the B. enteritidis, and 2 of the Paratyphoid A type ;
,, a child, 1 culture of the B. enteritidis ; and
,, the horse, 1 culture of the Paratyphoid A type.

It will be noticed from the above tables that the scrapings from the intestines yielded a larger number of positive results than the faeces. This has been observed by other workers in searching for the dysentery group of organisms, and is no doubt due to the fact that the bacilli are parasitic on the mucous surfaces of the intestines. Ford, in his paper,[10] states that amongst other bacteria the B. enteritidis of Gaertner has been isolated from the human caecum, and that it is practically never found in any other part of the intestines. Curiously enough the culture I obtained of the enteritidis group was from the small intestine of a child, the large intestine yielding no result. As regards the relative frequency of these bacilli in the large and small intestines it may be noted that in my experiments, both the large and small intestines of the various animals, regarded collectively, yielded nearly the same number of cultures of the enteritidis group, or 9 cultures from the small intestines and 10 from the large intestines. The same also applies to the Paratyphoid A group, 3 cultures being obtained from the small and 2 from the large intestines.

These tests being completed, the cultures of the organisms were tested as regards their pathogenicity, 2 c.cm. of a twenty-four-hours broth culture being injected intraperitoneally into a guinea-pig. This injection, in the case of the cultures of the B. enteritidis type proved uniformly fatal in a few hours. The cultures of the Paratyphoid A type proved, however, much less virulent, 75 per cent. of the guinea-pigs dying at the end of two to four days, the remaining 25 per cent. recovering. The cultures which did not kill guinea-pigs were therefore tested on mice, ½ c.cm. of a twenty-four-hours broth culture

being injected intraperitoneally, and with an invariably fatal result in from one to three days. No difference could be found between these two varieties of the Paratyphoid A type other than that shown by their virulence.

As was previously mentioned when describing the characteristics of the B. enteritidis group, a toxin is present in broth cultures of these organisms.

The bacilli of this group isolated by me from the intestines of animals were further subjected to the following test *re* toxicity. Two of the cultures were selected at random and grown on peptone beef broth at 37° C. for five days. The broth culture was then filtered through a Pasteur-Chamberland filter, and 2 c.cm. of the filtrate injected intraperitoneally into guinea-pigs, with the result that the animals died within twenty-four hours. A similar experiment was made with the toxin produced by the B. enteritidis of Gaertner (original strain), with the same result, proving that the bacilli of the enteritidis group obtained from the intestines of healthy animals are capable of producing a soluble toxin similar to that produced by other known members of the same group.

From the experimental tests upon the bacilli obtained from the intestines of animals, it will be seen that 21 of the organisms found agree biologically, culturally, and morphologically with the known members of the B. enteritidis group, and 10 with the members of the Paratyphoid A group. The sources of the cultures with which comparison was made were quite reliable. I am indebted to Professor Gaertner for two cultures of the B. enteritidis of Gaertner, to Professor van Ermengem for a culture of B. Aertrycke, to Professor Theobald Smith for cultures of hog cholera "Maryland" and "Arkansas," and to Professor J. McFadyean and Dr. Evans for cultures of their hog-cholera bacilli. The cultures of B. typhi murium and B. psittacosis were obtained from the Pasteur Institute, and a large number of the food-poisoning bacilli were obtained from Kral.

From the account of the foregoing experiments, it will be seen that bacilli of the B. enteritidis type were more frequently met with than bacilli of the Paratyphoid A type, and it occurred to me that this was probably due to the much greater virulence of the former, as proved by inoculation experiments. It was therefore determined to test their relative frequency in the normal intestine of animals by direct cultivation from the scrapings of the intestines without passage through guinea-pigs. By this means the proportionate increase of the more virulent bacilli was prevented, and their relative frequency could be more accurately determined. The colourless colonies were picked off from the plates, planted on dulcit broth, and incubated at 37° C. for twenty-four hours. Tubes giving acid and gas at the end of this time were put through the various sugar media and litmus milk, as in former experiments. Two experiments were made with pig's intestines. In the first, 1 colony out of the 95 isolated from the plates proved to be of the Paratyphoid A type, and none of the B. enteritidis type. In the second experiment, out of 38 colonies 25 were of the Paratyphoid A type, and none of B. enteritidis type. This tends to show that, although in experiments where passage through animals was used the B. enteritidis type appeared to be more common than B. paratyphoid A type, it is nevertheless not so common in the intestines of animals as the latter.

The absence of bacilli of the enteritidis group, in experiments where passage through an animal was not used, is readily explained by the small amount of the culture employed for the inoculation of the plates—namely, a platinum loopful of a broth culture for two plates. In this small amount of material they might easily escape detection, and yet, through their greater virulence, far exceed the others after their passage through an animal. In fact, it may be as difficult to detect bacilli of the B. enteritidis type by plating directly from a dulcit-broth culture as it is to detect the B. typhosus in drinking water.

Up to the present the cultures obtained from the intestines of healthy animals have been classified broadly, as of the enteritidis or of the Paratyphoid A type, no attempt having been made to define particular species. In the present state of our knowledge, it seems better to base such classification upon the agglutination reactions of the organisms, for although so-called cross agglutinations may occur, as in the case of hog cholera, and typhoid, as instanced by Theobald Smith,[7] they occur, as a rule, only in low serum dilutions. If high dilutions of serums are used—1 in 1,000 or 1 in 20,000—the bacilli generally refuse to react with heterologous serums, but continue to react with homologous serums. These serum reactions have been of great value in grouping the organisms in question, as well as in proving the identity of the bacilli isolated in various food poisoning epidemics, as instanced by Durham[11] in his account of the Chadderton outbreak, and again in his account of an epidemic in which 185 persons were affected.[12]

Perhaps one of the most complete classifications of the food-poisoning and paratyphoid bacilli based upon agglutination reactions is that given by Trautmann.[13]

The organisms are found by Trautmann to fall into one or other of five groups—namely:

A. *Enteritidis (Gaertner).*

1. Gaertner (Frankenhausen).
2. Van Ermengem (Morseeler).
3. Fischer (Haustedt).
4. Abel (Hamburg).

B. *Breslaviensis.*

1. Kaensche (Breslau).
2. Günther (Prov. Posen).
3. Lochmann (Giessen, "caseolyticus").
4. Trautmann (Düsseldorf).

C. *Hamburgensis.*

1. Schottmüller (Hamburg, Fall Seeman).
2. Bac. bremensis febris gastricae Kurth.
3. Hünermann (Saarbrüken-Coblenz).
4. Conradi u. Drigalski und Jürgens (Saarbrücken). Type B of Feyfer and Kaysers.

D. *Strassburgensis.*

1. Schottmüller (Hamburg)
2. Brion and Kayser (Strassburg)

} Type A of Feyfer and Kayser.

E. *Morbificans Bovis* (Basenau).

Agglutination Table of known Bacilli with—(1) Gaertner Serum; (2) Aertrycke Serum; (3) Serum of Rabbit Immunized with a Bacillus of the Enteritidis Group Isolated from the Large Intestine of a Pig; (4) Paratyphoid A Serum.

These serums caused a distinct reaction with their own bacilli in dilutions of 1 in 100,000.

Bacterium.	Gaertner Serum.		Aertrycke Serum.		B. from Pig's Intestine Serum.		Para-typhoid A Serum.	
	1 in 200	1 in 1,000	1 in 200	1 in 1,000	1 in 200	1 in 1,000	1 in 200	1 in 1,000
1. *Organisms of the Gaertner Type.*								
B. ent., Gaert., L.I.P.M.	3	3	0	0	0	0	—	—
B. Gaertner, Guy's...	2	2	0	0	0	0	—	—
B. Gaertner, van E.	3	3	0	0	0	0	—	—
B. typhi murium ...	2	2	0	0	0	0	—	—
B. Moorsele	3	3	0	0	0	0	—	—
B. psittacosis, No-card	3	3 (3, 1 in 100,000)	3	3 (3, 1 in 100,000)	3	3 (3, 1 in 100,000)		
B. Haustedt	3	3	0	0	0	0	—	—
Abel	3	2	0	0	0	0	—	—
B. Rennfleth... ...	3	3	0	0	0	0	—	—
B. icteroides... ...	0	0	0	0	0	0	—	—
2. *Organisms of the Aertrycke Type.*								
B. Aertrycke... ...	2	2	3	3	3	3	—	—
Hog cholera, Evans	0	0	3	3	3	3	—	—
Hog cholera, "Maryland"	0	0	2	2	2	0	—	—
Hog cholera, "Arkansas"	0	0	1	1	1	0	—	—
Hog cholera, "McFadyean"	0	0	2	1	2	1	—	—
B. Breslaviensis ...	0	0	3	2	3	2	—	—
Günther	0	0	3	2	3	3	—	—
3. *Organisms of the Paratyphoid Type.*								
B. paratyphoid A ...	0	0	0	0	0	0	3	3
B. paratyphoid B ...	0	0	0	0	0	0	0	0
B. paratyphoid, Brion and Kayser	3	2	3	0	0	0	3	3
4. B. morbificans, bovis	0	0	0	0	0	0	0	0
5. B. Wesenberg ...	0	0	0	0	0	0	0	0
6. Grünthal	0	0	0	0	0	0	0	0
B. typhoid, L.I.P.M.	2	2	0	0	0	0	0	0
B. Shiga (dysentery)	0	0	0	0	0	0	0	0

N.B.—1 = slight reaction; 2 = distinct reaction; 3 = complete reaction.

The following attempt was made to ascertain to which group, or groups, of organisms the cultures obtained by me from the intestines of animals belonged: Three rabbits were immunized—the first with B. enteritidis of Gaertner, the

second with B. Aertrycke (a food-poisoning bacillus of the Breslaviensis and Günther group), and the third with bacilli of the enteritidis group, which I had isolated from the large intestines of a healthy pig. The rabbits were immunized by subcutaneous injection of the respective cultures, and a monovalent serum was obtained, giving an agglutination reaction with its own bacillus in the dilution of 1 in 100,000. These three serums were then used to test the reactions given by the bacilli of the enteritidis and Paratyphoid A type that had been isolated from animals' intestines, the known bacilli of these two groups also being tested individually against the same serums. The results of these experiments are shown in the accompanying agglutination tables.

Organisms Isolated by Author from the Intestines of Healthy Animals Tested against Three Serums.

A.—*Bacilli of the Enteritidis Type giving no Reaction with Gaertner Serum.*

Source of Bacilli.	Gaertner Serum.		Aertrycke Serum.		B. from Pig's Intestine Serum.	
	1 in 200.	1 in 1,000.	1 in 200.	1 in 1,000.	1 in 200.	1 in 1,000.
Pig's faeces	0	0	3	3	3	3
Pig's small intestine (α) ...	0	0	3	3	3	3
Pig's small intestine (β) ...	0	0	2	1	3	3
Pig's small intestine (γ) ...	0	0	3	2	3	3
Pig's small intestine (δ) ...	0	0	2	2	3	3
Pig's large intestine (α) ...	0	0	3	3	3	3
Pig's large intestine (β) ...	0	0	3	3	3	3
Calf's large intestine (α) ...	0	0	3	3	3	3
Sheep's large intestine (α)...	0	0	3	3	3	3

B.—*Bacilli of Enteritidis Group giving a Partial Reaction to Gaertner Serum.*

Source of Bacilli.	Gaertner Serum.		Aertrycke Serum.		B. from Pig's Intestine Serum.	
	1 in 200.	1 in 1,000.	1 in 200.	1 in 1,000.	1 in 200.	1 in 1,000.
Pig's small intestine (ε) ...	2	2	3	3	3	3
Pig's small intestine (ζ) ...	2	2	3	3	3	3
Pig's large intestine (γ) ...	1	1	3	3	3	3
Bullock's large intestine (α)	2	0	2	2	3	3
Bullock's large intestine (β)	2	0	3	2	3	3
Bullock's large intestine (γ)	2	0	2	2	3	3
Bullock's large intestine (δ)	2	0	2	2	3	3
Sheep's faeces (α)	1	1	2	2	3	3
Calf's small intestine (β) ...	1	1	2	2	3	3

C.—*Bacilli of the Enteritidis Type giving a Complete Reaction to both Gaertner and Aertrycke Serum, like B. Psittacosis.*

Source of Bacilli.	Gaertner Serum.		Aertrycke Serum.		B. from Pig's Intestine Serum.	
	1 in 200.	1 in 100,000.	1 in 200.	1 in 100,000.	1 in 200.	1 in 100,000.
Child's small intestine (α)..	3	3	3	3	3	3
Pig's small intestine (η) ...	2	2	2	2	3	3

N.B.—1 = slight reaction; 2 = distinct reaction; 3 = complete reaction.

On comparing the agglutination table of the known food-poisoning bacilli with Trautmann's list it will be found that the results as regards the enteritidis or Gaertner group, and the Breslaviensis or Aertrycke group, are identical with my own, and it is with these two groups that the present research is most concerned, as all the bacilli of the enteritidis group isolated from animals' intestines reacted quite as well to the serum of the second rabbit (Aertrycke serum) as to the serum of the third rabbit immunized by one of the cultures obtained from the large intestine of a healthy pig. It is true that about half the cultures of the enteritidis bacilli reacted to a certain extent to the serum of the first rabbit (immunized against B. Gaertner), but in the higher dilutions it was shown that the Aertrycke serum had a much greater effect. In the same way the Aertrycke culture itself gave a moderate reaction with the Gaertner serum, and a complete reaction with the Aertrycke serum, in a dilution of 1 in 1,000.

These results, together with the previously-mentioned cultural reactions, indicate that 18 of the 20 cultures examined belong to the Aertrycke, or Breslaviensis, species of the enteritidis group. The remaining two cultures of this group, from the small intestine of a pig and from the small intestine of a child, were found to react equally well either to the Gaertner or to the Aertrycke serum, even in a dilution of 1 in 100,000. This peculiarity was also observed in the B. psittacosis, pointing to the conclusion that these two cultures were nearly allied to that bacillus. It will be noticed that the 10 cultures of the Paratyphoid A group gave no reaction with the three serums above mentioned. A fourth rabbit was therefore immunized with a culture of B. paratyphoid A of Schottmüller, and each of these cultures tested against the serum. None of the ten cultures, however, gave any reaction, thus giving no reaction at all with the four serums experimented with. It is certain that, according to their cultural reactions, they belong to the Paratyphoid A group, but in their serum reactions they do not agree with two known cultures of the group—Schottmüller's Paratyphoid A and the paratyphoid of Brion and Kayser. They belong possibly to yet another species of the same group.

The agglutination tests furnish one of the best laboratory methods of classifying the different organisms, and are much more delicate than the cultural methods, as is shown in the case of the four organisms of the hog-cholera group "Maryland," "Arkansas" of Theobald Smith, Evans's bacillus, and

McFadyean's bacillus, which all differ in their cultural reactions, but, as will be seen on reference to the table, react equally well to the Aertrycke serum. The agglutination reaction, however, does not necessarily indicate the specific virulence for different animals, the B. of mouse typhoid, for example, giving the same agglutinative reaction as the B. enteritidis of Gaertner, although the virulence of the mouse typhoid bacillus is, so far as we know, confined to mice and rats. The hog-cholera bacillus of McFadyean is virulent for pigs, but not for guinea-pigs. This specific virulence may possibly be acquired by passage through certain definite animals, but this is a matter for further research.

It will be observed that the cultures of the enteritidis group isolated from animals' intestines agree in agglutination reactions with the hog-cholera bacilli, as they are both agglutinated with Aertrycke serum; they also agree culturally with the hog-cholera bacilli of Evans, and "Maryland" of Theobald Smith.

It was decided to test, therefore, whether these bacilli from animals' intestines could produce hog cholera in the pig. A broth culture of bacilli of the Aertrycke type isolated from the large intestine of a pig being selected, 500 c.cm. of this culture, mixed with milk, was given to a young pig 4 months old, that being the most susceptible age. The animal, however, did not contract hog cholera. A second feeding experiment was tried with another young pig, a litre of a forty-eight hours' broth culture being administered. This produced violent diarrhoea and loss of appetite on the following day, which passed off in thirty-six hours, but on the animal being killed a week later no trace of the lesions peculiar to hog cholera was found in the intestines. The *post-mortem* examination was kindly performed by Professor McFadyean, of the Royal Veterinary College. The violent diarrhoea in the second feeding experiment seems a further proof of the relationship of these bacilli with those of the food-poisoning group.

General Conclusions.

1. There exist in the intestines of healthy animals organisms conforming morphologically and biologically to the enteritidis and Paratyphoid A types.

2. These organisms, as regards their agglutination reactions, fall into the following groups:

1. B. enteritidis, Aertrycke, or hog-cholera type.
2. B. enteritidis, psittacosis type.
3. B. paratyphoid A, unknown type.

3. The nomenclature adopted by different observers is unsatisfactory, but is based on the principal differentiating criterion we at present possess—namely, specific virulence.

My best thanks are due to Dr. Allan Macfadyen for many valuable suggestions during this research.

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OBSERVATIONS RELATING TO THE STRUCTURE AND GEOGRAPHICAL DISTRIBUTION OF CERTAIN TRYPANOSOMES.

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The following notes on trypanosomes which I have observed in certain mammals, birds, and goldfish obtained in the neighbourhood of Elstree in Hertfordshire appear worthy of publication, for the reason that our knowledge regarding the geographical distribution of trypanosomes is far from complete. No description has as yet been given of the trypanosomes found in bats by various observers, and a species encountered by me in the mole is, I believe, new.

I. Mammals.

Trypanosomes of Bats.

Laveran and Mesnil (1904, p. 104) state that trypanosomes have been observed by Dionisi in *Miniopterus schreibersii*, in other bats by Testi (1902) and Sambon (1904) in Italy, and by Durham (1900) in Parà; Donovan in Madras found trypanosomes in *Pteropus medius*. Hitherto no account has been published of the structure of bat trypanosomes[1].

Of eight bats caught here during the summer (June—July, 1904) (*Pipistrellus pipistrellus*) and examined shortly after death three showed trypanosomes in their blood.

[1] During the preparation of this paper for publication an account of trypanosomes occurring in bats in North Africa has been given by Ed. and Ét. Sergent (1905). They describe two forms, a small and a large trypanosome, the latter occurring rarely, and having been seen only in the fresh state. The small forms were found in 10 *Vespertilio kuhli* (Natterer) out of 26 examined, and in 7 *Myotis murinus* (Schreber) out of 35 examined. The large trypanosome was found in 2 *V. kuhli* (26 examined). The observers have named the small form *Trypanosoma nicolleorum* and the large one *T. vespertilionis*.

Numerous trypanosomes were found in the blood of one of the infected bats (a young female); the organisms disappeared from a hanging-drop preparation which was kept at 36° C. overnight. In the second bat a few trypanosomes were seen in the peripheral blood. The animal was etherised and the heart-blood examined but none could then be seen. In the third bat the trypanosomes were abundant in the blood. The skin of this bat was infested with fleas. Careful examination revealed no other external parasites on the skin.

A little of the blood from the last bat was inoculated intraperitoneally into a young white rat but an infection did not follow. Cultivation of the trypanosomes was attempted on rabbit-blood agar, kept at room temperature. At the end of 8 days numerous parasites were seen and one small clump of 2 or 3 members was suggestive of commencing multiplication. Unfortunately the medium became contaminated by a mould and the organisms soon disappeared.

It may be added that a specimen of a larger species of bat (*Pterygistes noctula*) showed no trypanosomes in its blood.

Structure of the Bat Trypanosome.

In a fresh preparation the movements of this trypanosome are very active, and seem to indicate a greater contortion than in the case of the rat trypanosome. It appears, moreover, to be considerably smaller than *Trypanosoma lewisi*. Preparations stained by Leishman's method show that it is in reality a small organism. Measurements were difficult on account of the almost invariably contorted position of the parasite in stained preparations. The length of one, which was in an extended position, was 16 μ (including the flagellum); the flagellum measured 8 μ. The breadth, including the membrane, near the macronucleus, was 1·5 μ.

In stained specimens the appearance of this trypanosome is quite characteristic, the large majority forming a circle or oval on account of the apposition of the anterior and posterior ends. Another distinctive feature lies in the close approximation of the macronucleus to the anterior end. The posterior extremity is pointed. The macro- and micro-nucleus stain deeply, while the protoplasm of the body of the parasite stains uniformly pale-blue—no granules being observable. The general appearance of the stained organism is such as to render its differentiation from the trypanosomes hitherto described a very easy matter.

Trypanosome of the Rabbit.

In a previous paper (1904) I described certain features of a trypanosome found accidentally in the blood of three tame rabbits, the parasite resembling very closely that of the rat. Microscopically it is impossible to distinguish them. Another similarity is found in the fact that the rabbit trypanosome is able to live for at least a month outside the body if kept at a comparatively low temperature.

Further observations have shown that tame rabbits are rarely infected; thus, 230 rabbits have been examined without discovering a single infected animal. Forty wild rabbits have, however, yielded 4 positive results. It may, therefore, be fairly assumed that the wild variety is more frequently infected than the tame, exactly as in the case of the rat. To ensure that the parasites should not be overlooked, in eleven instances 10 c.c. of blood were defibrinated and centrifugalized, but the results were again negative.

So far I have not been successful in transmitting the infection from the wild variety to tame rabbits by inoculation ; and attempts to infect white rats have also been unsuccessful.

A culture in rabbit-blood agar of trypanosomes from one of the wild rabbits has recently been obtained. After being kept for 16 days at room temperature a hanging-drop preparation was made and showed dividing forms, though not in great numbers. The largest number seen in any one field under a $\frac{1}{12}$ oil immersion lens was 12, arranged in groups of 5, 4 and 3 members; whereas in the original blood many fields had to be examined before one was observed. The forms seen were similar to those in cultures of *Trypanosoma lewisi.*

Trypanosoma lewisi.

Trypanosomes occur in wild rats in this locality with considerable frequency. No exact data are available but the proportion infected may be stated roughly as 30 %. In a lot of 6 rats captured in one place in the middle of December 1 full-grown and 4 young rats were found to be infected. The blood of 2 of the young rats contained numerous dividing forms, including rosettes consisting of small trypanosomes—one group being composed of 10 individuals. It may be noted that this stage is very rarely observed in wild rats; the only observers of it hitherto being Sivori and Lecler. In specimens stained by Leishman's method numerous forms arising from division were seen

similar to those observed in an artificially produced infection. Special attention was paid to the character of the rosettes in stained preparations in view of the recently published observations of MacNeal (1904) on the multiplication forms. MacNeal contends that the grouping by Laveran and Mesnil of the methods of division cannot be accepted. The latter observers classify the dividing forms into two groups, the 1st including those arising from unequal longitudinal division, the 2nd consisting of forms in which there is "a simultaneous division into several coequal daughter-cells, no mother-cell being distinguishable." The forms which I have observed in the specimens of the natural infection undoubtedly support the views held by MacNeal. In every rosette the flagellum of the parent cell can be easily distinguished, while on the other hand on no occasion have rosettes been seen which resemble the figures given by Laveran and Mesnil. The anomalous forms referred to by MacNeal, in which a long, slender extension of the posterior extremity occurs, were also noted. The significance of this does not appear to be known.

Mole (*Talpa europaea*).

The blood of a mole was examined a few hours after death. There were a very few trypanosomes present, which appeared to be similar in size and form to the rat trypanosome. The blood was pipetted into rabbit-blood agar, and kept at room temperature. 24 hours later one trypanosome with extremely active movements was seen in a hanging drop. Cultivation proved unsuccessful owing to contamination. Films of the blood were stained, but stained organisms could not be found. 3 moles captured in the month of January were examined with negative results.

Recently 5 other moles have been found to be infected, *i.e.* 6 altogether out of 20 examined (30 %). The parasites in every case were present in such small numbers that a satisfactory stained preparation has not yet been obtained. A white rat was inoculated intraperitoneally with the heart-blood of one of the infected animals, but no infection followed.

Field-Vole (*Microtus* [*Arvicola*] *agrestis*).

12 of these have been examined with negative results.

II. Birds.

Trypanosomes of Birds.

It may be recalled that trypanosomes in birds have been found by Danilewsky (1885–1889) in Russia, by Dutton and Todd (1903) in the Gambia, by Hanna and Donovan (1903) in India, and by Ed. and Ét. Sergent (1904) in Algeria. Laveran and Mesnil (1904, p. 353) describe them as occurring in the owl (*Syrnium aluco*) in France, but it is interesting to note that they record their failure to observe them in a great number of other birds in that country. Levaditi, however, has discovered a trypanosome in the blood of *Padda orizyvora* obtained in Paris. An account of the structure of this organism is given by Laveran and Mesnil, and inoculation experiments have been carried out quite recently by Thiroux (1904) in the Pasteur Institute. Schaudinn's (1904) conclusions on the relationship between trypanosomes and endoglobular parasites were based on observations of the blood parasites of *Athene noctua* in Rovigno.

The examination of the blood of birds in this district proved negative with regard to the occurrence of trypanosomes; on the other hand, these parasites were found in several cases in the bone-marrow. The birds were mostly obtained in the months of June, July and September, and preparations of the blood and marrow were made within an hour or two of death. The following Table gives the results in a succinct form.

Thus out of 67 birds examined 11 had trypanosomes in the bone-marrow (16·4 %). In those cases in which they were present a careful search was necessary in order to detect them, and, indeed, in stained preparations it was almost impossible to find a specimen. The blood was in every case examined for trypanosomes and intra-corpuscular parasites but on no occasion were these observed.

The trypanosomes found in the marrow showed active movements when examined in normal salt solution; movements from place to place did not occur to any extent. In a fresh specimen from a blackbird several refractive granules were seen in the middle of the body of the parasite. A trypanosome found in a swallow appeared to be smaller than one observed at the same time from a blackbird.

The discovery of a *Spirochaete* in the blood of a martin and a trypanosome in its bone-marrow is interesting in the light of Schaudinn's researches. In support of his views regarding the relationship between

Bird	Number examined	Date	Result
House-Martin; *Chelidon urbica* (Linn.)	1	June	Spirochaetes in blood; a few trypanosomes in bone-marrow.
Song-Thrush: *Turdus musicus* (Linn.)	6	June, July, Sept., Nov., Dec.	3 examined in June and July had trypanosomes in marrow.
			1 (28. 9) negative.
			1 (22. 11) blood negative; one trypanosome seen in fresh preparation of marrow.
			1 (19. 12) blood negative; one trypanosome seen in fresh preparation of marrow.
Blackbird; *Merula merula* (Linn.)	6	July, Sept.,	1 (15. 9) blood negative; one trypanosome seen in preparation of marrow.
		Nov., Dec.	1 (25. 11. 4) blood negative; fair number of trypanosomes in marrow. Fresh preparations of liver, spleen, ovary, kidney, all negative.
			1 in July; blood and marrow negative.
			1 (17. 11) ,, ,, ,, ,,
			1 (26. 11) ,, ,, ,, ,,
			1 (12. 12) ,, ,, ,, ,,
Swallow; *Hirundo rustica* (Linn.)	11	June, July, Sept.	1 (15. 9) a very few trypanosomes in marrow.
			5 in June—July; blood and marrow negative.
			3 (14. 9) ,, ,, ,, ,,
			2 (22. 9) ,, ,, ,, ,,
Chaffinch; *Fringilla coelebs*	1	26. 11. 4	Blood negative; a few trypanosomes in marrow.
Yellowhammer; *Emberiza citrinella*	1	16. 11. 4	,, ,, ,, ,, ,, ,,
House-Sparrow; *Passer domesticus* (Linn.)	27	June, July, Sept., Nov.	4 in June—July; blood and marrow negative.
			2 (15. 9) ,, ,, ,, ,,
			16 (21. 9) ,, ,, ,, ,,
			1 (9. 11) ,, ,, ,, ,,
			4 (16. 11) ,, ,, ,, ,,
Starling; *Sturnus vulgaris* (Linn.)	8	June, July, Sept., Nov., Dec.	5 in June, July, Sept.; blood and marrow negative.
			1 (28. 11) ,, ,, ,, ,,
			1 (12. 12) ,, ,, ,, ,,
			1 (19. 12) ,, ,, ,, ,,
Crow; *Corvus frugilegus* (Linn.)	4	June, July, Sept.	3, blood and bone-marrow negative.
			1, ,, ,, ,, ,, ,,
Jackdaw; *Coloeus monedula* (Linn.)	2	June, July	,, ,, ,, ,, ,,

these two organisms he mentions a similar instance, viz., the *Spirochaete* and *Trypanosome* found by Theiler (1903) in ordinary red-water and Rhodesian red-water fever (African Coast Fever). The spirochaete was present in considerable numbers in the martin's blood when examined immediately after death. In the fresh specimen the organism appeared to have several spiral twists (4 or 5), but in the stained preparation not more than two. In a film of the blood stained by Leishman's method the protoplasm has a pale-blue tint with no chromatin staining. The average length is 6·7 μ, breadth 1·5 μ. The spirochaetes were not present in the bone-marrow and there were no intra-corpuscular parasites.

III. Fishes.

Trypanosoma danilewskyi.

19 goldfish were examined, chiefly in the early months of the year. Trypanosomes were found in all, though in such small numbers that prolonged examination of the fresh specimen was usually necessary.

In a hanging-drop preparation the movements were exceedingly active and very contorted. The protoplasm had a granular appearance. With Leishman's stain the protoplasm of the body has a pale-blue colour, and contains small unstained spaces and chromatin granules. The macronucleus is situated near the middle of the body and stains more feebly than the micronucleus. As regards measurements the length of one was 38·4 μ, of another 32 μ, and of a third 48 μ: the breadth varied from 2 to 3 μ.

The blood of an infected fish was removed aseptically from the heart and pipetted into the condensation water of rabbit-blood agar. The tubes were then kept at room temperature. In 10 days a considerable number of trypanosomes were seen in a hanging-drop preparation. In shape they were curiously tadpole-like, contrasting with the slender form of the fresh specimen. Their movements were much less active than those in a fresh blood preparation. On the 11th day a group of 4 members was seen, and on the 12th day one with 5 individuals. On the 13th day the trypanosomes were much fewer. Sub-cultures were made but did not grow, though a considerable amount of inoculating material was added to the culture tubes. The shape of the trypanosomes, the feeble motility of their flagella, and the fact that they did not give rise to a sub-culture point to their being degeneration

forms, similar to those observed in cultures of *Trypanosoma lewisi* and *Trypanosoma brucei.*

Small leeches were occasionally found attached to the scales of the fish during the summer months, and as they were the only external parasite seen it is not unlikely (as has already been suggested) that the infection is conveyed by them.

The principal point of interest elicited by these observations lies in the fact that in a limited area so many animals of widely varying species should harbour trypanosomes. The following Table shows this clearly:

I. Mammals.

1. Bats:—(*a*) *Pipistrellus pipistrellus*, 8 examined, 3 positive.
   (*b*) *Pterygistes noctula*, 1 examined, negative.
2. Rats:—proportion of infected animals estimated at 30 %.
3. Wild Rabbits:—40 examined, 4 positive—(10 %).
4. Field-Vole (*Microtus agrestis*):—12 examined, all negative.
5. Mole (*Talpa europaea*):—20 examined, 6 positive—(30 %).

II. Birds.

67 examined, 11 positive—(16·4 %).
Trypanosomes were found in 6 out of 10 species.

III. Fishes.

19 Goldfish examined, all positive.

EXPLANATION OF PLATE VIII.

Fig. 1. *a—g*: Trypanosome of the bat—showing frequent contortion of the body of the parasite.

Fig. 2. Multiplication forms of *Trypanosoma lewisi* occurring in blood of naturally infected wild rat; the flagellum of the parent-cell is easily distinguishable.

Fig. 3. Spirochaete observed in blood of the house-martin; trypanosomes were present in the bone-marrow.

Fig. 4. Trypanosome of the rabbit—showing a close resemblance to *Trypanosoma lewisi.*

Fig. 5. Trypanosome found in blood of goldfish (*Tryp. danilewskyi*).

Fig. 6. Sketch of fresh preparation of trypanosome found in the bone-marrow of the blackbird.

In conclusion, the more noteworthy facts recorded above may be summarised as follows:

1. The trypanosome of the bat seems to be sharply defined in

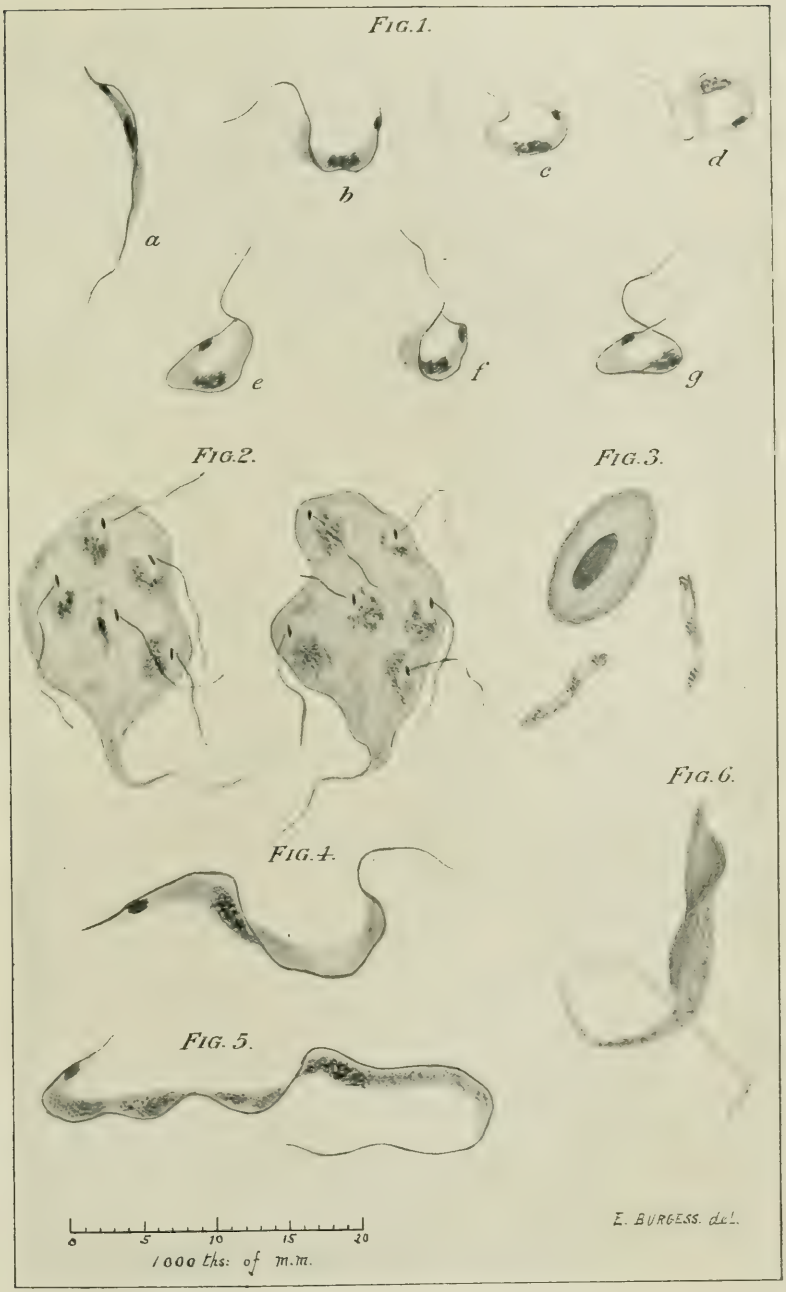
FIG. 1.
a
b
c
d
e
f
g
FIG. 2.
FIG. 3.
FIG. 4.
FIG. 5.
FIG. 6.
0 5 10 15 20
1000 ths: of m.m.
E. BURGESS. del.

several respects from trypanosomes hitherto described; chiefly from its small size and from its characteristic looped appearance in the stained specimen.

2. The wild rabbit, as in the case of the rat, is more frequently infected than the tame variety. Cultivation of the trypanosome for one generation at least presents no difficulty.

3. Blood-films from 2 wild rats with a natural infection of *Trypanosoma lewisi* in the stage of multiplication have been examined. The forms observed support the contention of Wasielewski and Senn and MacNeal that the mode of multiplication can be traced in every instance to an unequal longitudinal division.

4. A new species of trypanosome has been found in the blood of the mole.

5. Trypanosomes have been observed not infrequently in this locality in the bone-marrow of certain common birds but not in their blood.

6. No intra-corpuscular parasites have been observed in any of the birds examined.

7. In the case of one bird (*Chelidon urbica*, Linn.) a spirochaete in the blood was associated with a trypanosome in the bone-marrow.

I wish to express my thanks to Mr Oldfield Thomas, F.R.S., of the British Museum, who kindly identified the species of bats for me.

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ON THE RELATIONSHIP OF THE PSEUDO-DIPHTHERIA TO THE DIPHTHERIA BACILLUS.

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The extreme diversity of opinion as to the identity of the Klebs-Loeffler bacillus and the bacillus of Hofmann, and the importance of the subject from the public health standpoint, make it desirable to bring forward any facts which may assist in arriving at a solution of the problem.

For some years after the two organisms were recognised their morphological and cultural characteristics served as a basis for upholding or denying any essential differences between them. In recent years, however, it has followed as a natural outcome of the study of immunity that the methods used for investigating the problems of immunity have been applied in this particular instance, since it is possible to bring about a specific reaction even in the case of closely related bodies. The more important researches carried out on these lines may be briefly mentioned.

Spronck (1896) injected large doses of pseudo-diphtheria cultures subcutaneously into guinea-pigs, and found that the local reaction produced was not influenced by the subsequent injection of diphtheria antitoxin. Glücksmann (1897) ascertained that immunisation of animals with cultures of the Hofmann bacillus did not confer any protection against diphtheria bacilli injected later. Lambotte (1902) prepared a serum which he found contained a "substance sensibilisatrice" for pseudo-diphtheria bacilli. The results of testing this serum on different strains led him to believe that these were very closely related, if not identical organisms—the specific reactions being similar in each case. He found also that there was a certain amount of fixation of this

"substance sensibilisatrice" by Klebs-Loeffler bacilli. It may be remarked that Lambotte employed the Bordet-Gengou test for the fixation of the sensitising substance.

Several observers have attempted to solve the question by means of agglutination experiments but with conflicting results. Lesieur (1901) concludes from his experiments that pseudo-bacilli do not behave otherwise than true diphtheria bacilli towards a specific serum *in vitro*. Gordon's (1903) results showed considerable variations, even in the case of different strains of diphtheria bacilli; depending on whether the bacilli used for obtaining the agglutinating serum, and those used for the tests, were recently isolated or not. Lubowski (1900) immunised a goat with an avirulent diphtheria strain. The serum agglutinated virulent diphtheria bacilli and the avirulent organism but not Hofmann's bacillus.

The experiments about to be described were performed with the purpose of determining whether substances are present in pseudo-diphtheria filtrates which, when inoculated into animals in large amounts, lead to the production of an antitoxic serum for diphtheria toxin.

The work which has been done in this direction hitherto is inconsiderable. With regard to the production of a toxin, the non-pathogenicity of Hofmann's bacillus, *i.e.* the absence of a toxin producing acute symptoms, remains in the opinion of the majority of observers its distinguishing feature. It may be noted, however, that Ruediger (1903) has described pseudo-diphtheria organisms obtained from the throats of scarlet fever patients, which proved virulent to guinea-pigs. Antidiphtheria serum did not give any protection against these bacilli. Hamilton (1904) has isolated similar organisms from the throat in various diseased conditions and on one occasion from a normal throat. On the other hand, Graham-Smith (1904) states that while investigating an outbreak of diphtheria at Cambridge he failed to meet with organisms corresponding to the type described by Ruediger. Lesieur alleges that certain strains of pseudo-diphtheria bacilli, although non-virulent for guinea-pigs in ordinary doses, can nevertheless cause fatal paralyses similar to those due to diphtheria toxin provided that large doses of recently isolated cultures be used, or smaller doses of bacilli rendered artificially active by a method devised by himself. He evidently appears to consider that the paralysing substance is a very similar body, if it is not indeed identical with the toxone in diphtheria toxin. Several years ago Salter (1899) in a communication which is

frequently quoted described some interesting experiments, apparently showing that in filtrates of the Hofmann bacillus substances exist which are identical with the toxoids present in diphtheria toxin. Cobbett (1903) states that he has repeated these experiments without being able to confirm them. Hewlett (1904) also has been unable to obtain corresponding results.

I have carried out experiments in two directions in order to test this point:—

(1) by adding varying quantities of pseudo-diphtheria filtrates to toxin-antitoxin mixtures, and

(2) by immunising horses with large quantities of the filtrates and examining the serum afterwards for antitoxin.

The cultures used comprised 11 races. No. 1 was isolated from a throat-swab which was forwarded for diagnosis. It had been sub-cultivated on serum during a long period, at least 2 years. The remaining strains were recently isolated from swabs:—

1. From nose of patient suffering from throat diphtheria.
2. Throat of same patient.
3. Diphtheritic throat.
4. Throat of a case of scarlet fever with a deposit on the tonsils diphtheritic in appearance.
5. Throat of a case of suspected diphtheria.

6., 7., 8., 9., 10. were isolated from the throats and noses of boys from a school in which an outbreak of diphtheria occurred; the number of cases in which the Klebs-Loeffler bacilli were found was comparatively small.

All these strains had the following characters. They stained by Gram's, but not by Neisser's method. They gave an alkaline reaction to litmus when grown in glucose broth. Preparations from cultures grown for four days in alkaline broth at 36° C. when stained by Loeffler's methylene blue showed uniformly and deeply stained short rods arranged in a parallel manner. Involution forms were rarely seen even in cultures several weeks old. 5 c.c. of bouillon cultures grown for three days at 36° C., beyond slight local reactions at the site of injection, gave rise to no effects in guinea-pigs.

A large volume of culture was obtained by growing No. 1 bacillus in Erlenmeyer's flasks, each containing 200 c.c. of alkaline broth prepared in the usual manner for the production of diphtheria toxin. Another

quantity was grown in Martin's pig-stomach bouillon at 36° C. for ten days. The cultures developed a thick, opaque, slightly wrinkled membrane, of a greyish-white colour on the surface, exactly resembling that seen in diphtheria cultures; the broth was, however, somewhat turbid. The cultures were filtered through a Pasteur-Chamberland filter after growing for 10 days.

A mixed filtrate of the other 10 races was prepared by growing each separately after several sub-cultures in alkaline broth tubes. One lot was allowed to grow for nine days, the remaining flasks being filtered after 15 days' growth. Most of the cultures formed good membranes on the surface of the medium, and all had the peculiar, slightly offensive odour characteristic of diphtheria cultures.

The following Tables set forth the experiments carried out with a view to ascertaining whether toxoids were present in the filtrates. The filtrate was added first to the unit of antitoxin, the mixture being then placed in the incubator for fifteen minutes before adding the toxin. In this way any toxoid which might be present had an opportunity of combining with the antitoxin, thus preventing a corresponding amount of toxin from entering into combination.

All the mixtures were made up to the same bulk with tap-water. The toxin used was a test-toxin employed for testing antitoxic sera, the L+ dose being 0·14 and the Lo dose being 0·08 c.c.

It will be noted that in Table I the results are somewhat irregular in the case where 0·66 c.c. of toxin was given. This can doubtless be explained by the circumstance that the amount of toxin approximates so closely to the L+ dose. In the control experiment with 0·66 c.c. toxin the animal died on the 8th day, but in tests with the same amount of toxin carried out for another purpose the guinea-pig died in one case on the 4th day, and yet another on the 5th day. Differences in the resistance of different animals have also to be taken into account. With regard to the other tests in this table, although in one or two instances the animals which received the pseudo-diphtheria filtrates suffered a greater loss of weight than the controls, it is obvious that the difference comes within the limits of the errors of experiment. In Table II amounts of toxin more nearly approaching the L+ dose were selected. The results of this experiment show clearly that no evidence is forthcoming of there being any difference in the amount of free toxin present in the mixtures.

The dose of toxin given in the experiments set forth in Tables III and IV was such that toxone effects became manifest in 3 or 4 weeks.

TABLE I.

Testing Toxin – Antitoxin Mixtures + Pseudo-diphtheria Filtrate of No. 1 Bacillus, showing result of addition of the Filtrate to amounts of Toxin varying from a nearly L + to a nearly L° dose

Guinea-pig	Weight grams	Test	Weight of Guinea-pig on successive days after injection							
1	250	1 I. E. + 0·66 c.c. T_5	255 t	240 vl	218 vl	210 vl	200 l	193 vl	195 vl	dead 22nd day
2	258	,, + ,, + 1 c.c. Ps. F.	248 l	235 vl	220 vl	dead				
3	255	,, + ,, + ,,	245 m	220 vl	205 vl	185 vl	170 vl	173 vl	170 vl	dead 9th day
4	250	,, + ,, + ,,	225 l	220 vl	200 vl	dead				
5	255	,, + ,, + ,,	258 m	255 vl	253 vl	230 vl	dead			
6	255	,, + ,, + 0·5 c.c. Ps. F.	230 vl	215 vl	210 vl	198 vl	205 vl	205 vl	185 vl	recovered
7	255	,, + ,, + 0·25 c.c. ,,	258 m	235 vl	240 vl	225 l	215 vl	215 vl	215 vl	dead 22nd day
8	250	,, + 0·64 c.c. T_5	258 s	250 vl	240 vl	230 vl	230 l	215 vl	205 vl	dead
9	255	,, + ,, + 0·5 Ps. F.	255 m	260 l	250 vl	235 l	240 l	240 l	225 l	
10	250	,, + ,, + 0·25 c.c. Ps. F.	238 m	230 l	228 vl	225 m	235 l	235 l	225 l	dead 22nd day
11	248	,, + 0·62 c.c. T_5	265 t	242 l	230 vl	225 l	220 l	225 vl	235 vl	
12	255	,, + ,, + 0·5 c.c. Ps. F.	240 l	230 vl	220 vl	220 l	215 l	215 l	205 l	
13	250	,, + ,, + 0·25 c.c. ,,	240 m	235 m	238 l	245 s	240 s	230 s	220 s	
14	250	,, + 0·60 c.c. T_5	240 t	240 s	225 l	215 l	220 l	215 m	215 m	
15	250	,, + ,, + 0·5 c.c. Ps. F.	240 m	225 l	218 l	215 m	220 s	210 m	200 m	
16	250	,, + ,, + 0·25 c.c. ,,	235 s	225 m	235 l	235 m	235 m	230 s	220 s	
17	255	,, + 0·56 c.c. T_5	245 n	240 s	225 l	220 l	220 m	220 t	215 m	
18	250	,, + ,, + 1 c.c. Ps. F.	215 m	200 l	195 vl	195 l	200 m	200 m	195 s	
19	245	,, + ,, + 0·5 c.c. ,,	230 s	220 l	220 m	225 s	225 s	220 t	220 s	
20	245	,, + 0·52 c.c. T_5	245 n	230 t	228 s	215 m	215 m	215 t	215 t	dead 11th day
21	250	,, + ,, + 1 c.c. Ps. F.	245 s	228 m	235 s	235 s	225 t	220 t	223 n	
22	250	,, + ,, + 0·5 c.c. ,,	240 t	230 s	225 t	228 t	225 t	220 t	228 t	
23	260	,, + 0·50 c.c. T_5	258 n	255 t	265 t	245 t	260 t	255 t	255 n	
24	248	,, + ,, + 1 c.c. Ps. F.	240 m	230 m	225 t	235 t	230 t	220 t	215 t	
25	255	,, + ,, + 0·5 c.c. ,,	275 t	250 n	235 t	235 t	240 n	240 n	255 n	

Note:—1 I. E. = 1 unit of Antitoxin (Ehrlich). T. = Toxin. Ps. F. = Pseudodiphtheria Filtrate.

The Toxin was diluted 1 : 5 with water for convenience of testing; the amount of Toxin represents amounts of this dilution in this and the following Tables.

The letters "n," "t," "s," "m," "l," and "vl," refer to the size of the local reaction, and indicate respectively that there was no local reaction; a trace of reaction; and a small; medium; large; and very large reaction.

TABLE II.

Testing Toxin – Antitoxin Mixtures + Pseudo-diphtheria Filtrate (10 *races grown separately and Filtrates mixed*), *showing result of addition of the latter to amounts of Diphtheria Toxin approaching the L + dose.*

Guinea-pig	Weight grams	Test	Weight of Guinea-pig on successive days after injection							
1	275	1 I. E. + 0·69 c.c. T_5	260 l	250 vl	230 vl	230 vl	—	dead	—	—
2	260	,, + ,,	260 m	255 l	230 l	225 vl	dead	—	—	—
3	255	,, + 0·67 c.c. T_5	260 m	250 l	230 l	210 l	dead	—	—	—
4	250	,, + ,,	240 m	235 vl	210 vl	215 vl	—	180 vl	dead	—
5	265	,, + 0·69 c.c. T_5 + 2·5 c.c. Ps. F. 9 days	255 l	255 vl	225 vl	225 vl	—	dead	—	—
6	260	,, + ,, + ,, ,, 15 days	265 m	270 vl	250 vl	250 vl	—	215 vl	dead	—
7	255	,, + 0·67 c.c. T_5 + 2·5 c.c. Ps. F. 9 days	250 m	250 vl	255 vl	250 vl	—	250 vl	250 vl	265 vl
8	250	,, + ,, + ,, ,, 15 days	Paralysis pronounced on 28th day; dying on 33rd day; dead next day.							
			230 m	215 vl	190 vl	180 vl	—	dead	—	—

Table III.

Testing Toxin – Antitoxin Mixtures + Pseudo-diphtheria Filtrate (mixed Filtrates of 10 races), showing effect of addition of latter to amounts of Toxin representing paralysing dose.

Guinea-pig	Weight grams	Test	Result
1	275	1 I. E. + 0·65 c.c. T_5	Died with paralytic symptoms on the 28th day.
2	265	,, + ,, ,,	Marked paralysis on 30th day, but gradually recovered thereafter.
3	270	1 I. E. + 0·65 c.c. T_5 + 2·5 c.c. Ps. F.	Marked paralysis on 23rd day; died next day.
4	260	,, + ,, + ,, ,,	Paralysis on the 23rd day; died on 28th day.
5	250	,, + ,, + ,, ,,	Died on 23rd day.

Table IV.

Repetition of Experiment given in Table III.

Guinea-pig	Weight grams	Test	Result
1	260	1 I. E. + 0·65 c.c. T_5	Died on 8th day. P.M. Toxaemia.
2	260	,, + ,,	Showed commencing paralysis on 16th day, which became very pronounced on 21st day; died on 24th day.
3	260	,, + ,,	Showed marked paralysis on 20th day; died on 21st day.
4	250	,, + ,, + 2·5 c.c. Ps. F.	,, ,, ,, ,, 24th day; ,, 28th day.
5	250	,, + ,, + ,, ,,	,, ,, ,, ,, 22nd day; ,, 24th day.
6	250	,, + ,, + ,, ,,	Died on the 11th day.

The results are not conclusive of any marked difference in the paralytic effects produced.

Taken as a whole these experiments may be interpreted as indicating that no toxoids were present in the filtrates of the pseudo-diphtheria bacilli examined.

Immunisation with Filtrates of the Pseudo-Diphtheria Bacilli.

Another method of testing the point was tried. If toxoids be really present as a product of metabolism of the Hofmann bacillus it might be expected that immunisation of a suitable animal with their products ought to lead to the production of an antitoxin in the animal's serum. The validity of this contention will be discussed later.

The process of immunisation was carried out in the following way:

A horse "A" which had not been previously immunised, received doses of pseudo-diphtheria filtrate of No. 1 bacillus, the procedure being exactly the same as in a diphtheria immunisation. Beginning with 3 c.c. it received as a final dose 5 weeks later 1 litre; 3,200 c.c. being given altogether. Before the injections were begun the serum of the horse was tested for normal antitoxin. It was found that it did not contain 1/3 unit per c.c. Ten days after the final injection it was again tested and again was found not to contain 1/3 unit.

A month or two later this horse received 1/10 c.c. of a diphtheria toxin, the M.L.D. of which for guinea-pigs of 250 grammes was 1/100 c.c. No further injections were given until 10 days later. A sample was taken on this day and the serum was again tested. It was found to contain 1 unit per c.c. At the end of the ordinary diphtheria immunisation the antitoxic value of the serum was low (below 100 units per c.c.) and in subsequent immunisations never rose above 400 units per c.c. It may be noted that the morning after a dose of 450 c.c. of the filtrate had been given the horse showed a considerable local reaction.

A second horse "B" was treated in a similar manner. He received 2150 c.c. of the mixed filtrates (10 races grown for 15 days). Two days after the last injection of 1 litre, a litre of the 9 day filtrate was given. The period of immunisation extended over 20 days. Ten days later a sample was taken. Before the injections were commenced the serum was tested and was found to contain no normal antitoxin, *i.e.* not 1/4 unit per c.c. The serum of the sample taken after the immunisation also did not contain 1/4 unit per c.c. The horse was then

given 1/100 c.c. of a diphtheria toxin whose M.L.D. for guinea-pigs was 1/400 c.c. After an interval of 10 days, blood was withdrawn, and the serum again found to have less than a 1/4 unit per c.c. Four days later 1/20 c.c. was given intramuscularly. It was intended to obtain blood for the purposes of a test at the end of 10 days, but unfortunately the horse died suddenly; the cause of death apparently having no connection with the previous treatment.

On the evening after 150 c.c. of the filtrate was administered the horse had a temperature of 104·4° Fahr. and a small swelling the size of the fist over the site of injection. After 300 c.c. he had a local reaction with an oedematous swelling in the brisket and stiffness in walking. The dose of 1 litre of the 15 days' filtrate caused a moderate local swelling accompanied by oedema in the brisket. The symptoms, however, were not nearly so severe as those usually observed during a diphtheria immunisation.

A third horse "C" was inoculated with 650 c.c. of the 15 days' filtrate given by rapid stages, and as a final dose, 1,100 c.c. of the nine days' filtrate. The period of immunisation lasted only nine days. The serum before beginning the treatment did not contain 1/4 unit, and ten days after the last dose was given, again did not have an antitoxic value of 1/4 unit per c.c. The horse was then injected on successive days with 1/100, 1/50, 1/10, 1/2, 1·25 and 3 c.c. of a toxin whose M.L.D. for guinea-pigs was 1/300 c.c. The blood was sampled after an interval of ten days, and the serum was found to contain 1 unit per c.c., but not five units. In the evening after 150 c.c. of the pseudo-diphtheria filtrate had been given the horse had a moderate local reaction. He felt the last dose (1,100 c.c.) considerably, fed badly for a few days, and had a large local reaction.

It is evident from these experiments that the filtrates employed were not capable of producing an antitoxin to diphtheria toxin. Before the conclusion is drawn that no toxoids were present in the filtrates the question must be faced as to whether toxoids alone can give rise to an antitoxin. At the Thirteenth International Congress of Medicine at Paris in 1900 Ehrlich made several observations bearing upon this point which leave no doubt that he believed that they are able to do so. While referring to his own views on the constitution of diphtheria toxin he stated that it was possible to provoke a production of an antibody not only by utilising toxins but also toxoids. As a result of later investigations in conjunction with Morgenroth on the production of isolysins Ehrlich (1900) found that before such an antibody could be

obtained very large quantities of blood must be injected; the reason being that in the blood of the same species elements corresponding to toxophore groups are absent. He considers that what he calls an "ictus immunisatorius" is essential. Wassermann at the International Congress of Hygiene at Brussels in 1903 discussed the matter at some length. His conception of the mechanism of antibody-production may be briefly stated thus:—He asserts that he has never succeeded with quite non-toxic "toxins," *i.e.*, toxins with haptophore groups only, in obtaining a really high antitoxic serum. In order to bring about an "Abstossung" of the receptors a second factor besides the combination of the haptophore groups to the cells is necessary, viz., the stimulus (Reiz) to the cell which is the function of the toxophore groups. He points out, however, that the possibility of immunising with toxoids is beyond question, and gives as an example the basal immunity conferred on guinea-pigs and mice against tetanus by using non-toxic modifications: the only possible method indeed of producing an immunity in these animals against diphtheria and tetanus toxins. Bruck (1904) in a recent paper describes experiments which he thinks strengthens the position taken up by Wassermann. He obtained two tetanus toxins, one practically non-toxic for mice, and the other feebly toxic. He proved the presence of toxoids in these toxins by finding that they were still able to neutralise antitoxin. He then proceeded to immunise rabbits, giving doses up to 1 c.c. of each. In the case where the non-toxic substance was used no antitoxin was produced, while in the other case, a small amount of antitoxin was found in the serum of the rabbit. The objections can, I think, be reasonably adduced that the total quantities of the toxins given were very small,—Ehrlich's isolysin experiments may be recalled in this connection,—and that the rabbit is not perhaps a very suitable animal for the production of an antitoxin. Bruck believes that a slight stimulus is necessary in addition to the action of the haptophore groups for the production of an antibody.

Von Behring's (1904) views on this subject are founded on a very large practical experience. In immunising horses in order to obtain tetanus antitoxin he now uses toxins whose "direct toxic value" has almost, if not completely disappeared, but which have retained their "indirect toxic value," *i.e.*, their power of neutralising antitoxin. He refers to a tetanus toxin which, although quite non-toxic for mice, can be used for immunising horses without the slightest risk, and which in a short time confers upon them a high degree of immunity with a considerable production of antitoxin. Before he recognised this fact he had

empirically adopted the method of adding trichloride of iodine to his toxins in order to bring about a similar result.

The weight of von Behring's authority on this point, together with the fact that Wassermann does not deny that a small amount of antitoxin can be produced by toxoids alone, is confirmatory of the belief that no toxoids were present in the pseudo-diphtheria filtrates used in the immunisation experiments described above. This view gains support from the circumstance that after a small amount of diphtheria toxin was given the antitoxic values of the serum of the horses "A" and "C" were not above the average. It might have been supposed, otherwise, that the receptors produced in abundance by toxoids but remaining attached to the cells would have been set free in the serum when a stimulus provided by even a small dose of toxin was supplied.

In conclusion it may be stated that the two sets of experiments carried out combine to justify the opinion:

(1) that no substances capable of neutralising diphtheria antitoxin are present in filtrates of pseudo-diphtheria bacilli;

(2) that the results of the immunisation of horses with large quantities of the filtrates make it apparent that they do not contain substances capable of stimulating the production of an antitoxin to diphtheria toxin.

It is scarcely necessary to add that if this be the case the differences between the two organisms are accentuated, thereby diminishing the probability that they stand in a close relation to each other. The whole question has a certain interest clinically, since it shows that in cases where Hofmann's bacillus is associated with the Klebs-Loeffler bacillus no toxoids are elaborated by the former which might do harm by combining with antitoxin administered therapeutically.

My thanks are due to Dr Fletcher, Ham Green Hospital, Bristol, and to Dr A. T. MacConkey, for supplying me with the material from which the cultures were isolated. I am also indebted to Dr George Dean for practical suggestions which have been of value in carrying out the experiments.

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ON THE FORMATION OF FATTY ACIDS FROM LACTIC ACID WHEN FUSED WITH CAUSTIC ALKALIES.

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Nearly thirty years ago, in a series of studies on ferment action, Hoppe-Seyler[1] drew attention to the similarity between the chemical changes in putrefaction and those brought about by alkalies. In his study of these changes he found that lactic acid, when heated with alkalies, gave rise to the formation of hydrogen and of acids of the acetic acid series. Among these he claimed to have detected besides butyric and caproic, other higher fatty acids, and thus to have obtained an indication of the way in which fat may be made from carbohydrates in plants and animals.

Hoppe-Seyler's account of his experiments is short, and as regards the formation of the higher fatty acids is not altogether convincing. In view of this and of the importance of the question, it seemed desirable to repeat and extend the experiments, and to determine what fatty acids are formed by the action of alkalies on lactic acid. This I have done at the suggestion of Dr Leathes, who has also given me much helpful advice. I wish to express to him my sincere thanks.

Fusion of calcium lactate with soda lime.

The calcium lactate used in the experiments was obtained from Kahlbaum, and on examination was found to be free from calcium butyrate.

Portions of 75 grms. of this salt and 150 grms. of soda lime were ground up together and heated in a flask connected with an air condenser in a bath of fusible metal. As the temperature was raised water was given off; at 260–270° (measured in the bath) the evolution of hydrogen commenced and became very rapid as the temperature was

[1] *Zeits. f. physiol. Chem.* II. p. 1. 1878. III. p. 351. 1879.

raised still further. The heating was continued at 290–300° until the evolution of gas ceased. When cold the contents of the flask were extracted several times with hot water and filtered. The residue which was insoluble in water was digested for some time on the water-bath with dilute sulphuric acid and the resulting solution filtered and distilled. The distillate contained no volatile acids, hence in the later experiments the insoluble residue was not further treated in any way.

The solution obtained on extraction of the fused mass with water was acidified with sulphuric acid and distilled. The strongly acid distillate was neutralised with sodium hydrate and evaporated to dryness. The yield of sodium salts of volatile acids from 75 grms. calcium lactate was 15 grms. The residual liquid in the distilling flask contained a small amount of oil. This was extracted with ether and examined separately.

Examination of volatile acids. 140 grms. of the sodium salts of the volatile acids were decomposed with a slight excess of sulphuric acid (1 part acid to 2 parts water) and the solution extracted several times with ether. The aqueous portion was distilled, and in the distillate the presence of a small amount of formic acid was detected by its reducing action on silver nitrate. The ether solution was dehydrated over anhydrous sodium sulphate, the ether removed on the water-bath and the residual acids distilled. Fractions were collected between the following temperatures:

	Boiling point	Weight		Boiling point	Weight
1	Below 125°	35 grms.	4	145°—155°	1·5 grms.
2	125°—135°	8 ,,	5	155°—170°	1 ,,
3	135°—145°	3 ,,	6	Residue	1·5 ,,

The first two fractions were shown to consist principally of acetic acid by heating with alcohol and sulphuric acid, when a considerable quantity of ethyl acetate was obtained which boiled at 77°.

3rd Fraction. A portion of this was converted into the calcium salt and analysed.

0·8953 gm. gave 0·2842 gm. CaO = 23·63 % Ca
Calculated for calcium acetate = 25·32 % Ca
Calculated for calcium propionate = 21·54 % Ca.

The acid is thus a mixture of acetic and propionic acids.

4th Fraction. (Boiling point 145–155°.) The molecular weight of this was determined by titration with standard potassium hydrate.

0·4743 gm. required 10·95 c.c. KOH (1 c.c. = 0·0316 gm. KOH),
∴ Mol. wt. = 76·8.

The molecular weight of propionic acid is 74, hence this fraction contains a small amount of acid of higher molecular weight than propionic acid.

5th Fraction. (Boiling point 155–170°.) This was completely soluble in water but was precipitated on adding calcium chloride. It had a smell resembling that of isobutyric acid. The barium salt was prepared and analysed.

0·7241 gm. gave 0·5503 gm. $BaSO_4$ = 44·68 % Ba
Calculated for $(C_4H_7O_2)_2Ba$ = 44·04 % Ba.

In order to determine whether the acid was normal or isobutyric acid the calcium salt was prepared and the water of crystallisation estimated.

0·1428 gm. on heating at 140° lost 0·0190 gm. water = 13·31 % H_2O
Calculated for $(C_4H_7O_2)_2Ca.H_2O$ = 7·76 % H_2O
Calculated for $(C_4H_7O_2)_2Ca.H_2O$ = 14·40 % H_2O.

Calcium *n* butyrate crystallises with one molecule of water, whereas calcium isobutyrate crystallises with five molecules of water. Since calcium *n* butyrate is more soluble in cold water than hot, a saturated solution of the calcium salt was made at 0° and then heated, but no deposition of the calcium salt occurred. From the value obtained for the amount of water in the crystallised calcium salt it was thought that the acid was probably a mixture of butyric and isobutyric acids. To confirm this, calcium butyrate and isobutyrate were mixed in such proportions that the resulting mixture contained 13·31 % of water of crystallisation. A saturated solution was made at 0°, but on warming did not deposit calcium butyrate. The acid obtained as the fifth fraction is therefore most probably a mixture of butyric and isobutyric acids.

The Residue. This was dark brown in colour and contained small particles of tarry matter which were separated by diluting with petroleum ether and filtering. The filtrate separated into two portions. The lower portion, insoluble in petroleum, was slightly soluble in water, took up bromine in the cold, and hence was partly unsaturated. The barium salt was prepared and analysed.

0·2178 gm. gave 0·1184 gm. $BaSO_4$ = 31·97 % Ba.

Barium caproate contains 37·32 % Ba, hence the acid is of higher molecular weight than caproic acid. Being insoluble in petroleum it probably contains hydroxyl groups, and is therefore not a fatty acid.

The acid which was soluble in petroleum was obtained by evaporation

of the solution in the water-bath. It was moderately soluble in water and combined with bromine in the cold to a slight extent, being thus partly unsaturated. The barium salt was prepared and analysed.

0·3596 gm. gave 0·2280 gm. $BaSO_4$ = 37·20 % Ba
Calculated for barium caproate = 37·32 % Ba.

Since the acid is partly unsaturated it cannot be wholly caproic acid, but is probably a mixture of acids.

Non-volatile acid. The small amount of oil which was invariably left in the distilling flask after the removal of the volatile acids in a current of steam was extracted with ether and the ether removed in the water-bath, the residual oil was shaken up with water to remove lactic acid, and dried. From 700 gm. calcium lactate 0·55 gm. was obtained. The substance was unlike fatty acid in appearance, was resinous, and heavier than water. The barium salt was almost completely soluble in water, only a very small portion remaining undissolved. On analysis,

$$0{\cdot}5510 \text{ gm. required } 10{\cdot}1 \text{ c.c. } \frac{N}{2} \text{ NaOH} = 0{\cdot}202 \text{ gm. NaOH.}$$

Assuming the acid to be monobasic the molecular weight is thus 109.

Fusion of calcium lactate with caustic potash and magnesia.

A series of experiments similar to the above were carried out, using a mixture of equal parts caustic potash and magnesia instead of soda lime. The results were almost identical, except that a smaller yield of volatile acids was obtained. The butyric acid fraction was a mixture of the normal and iso-acids, as the calcium salt contained water of crystallisation almost equivalent to three molecules.

The residue obtained from the distillation of the volatile acids was partly unsaturated, although the molecular weight was very near to that of caproic acid.

The non-volatile acid was not fatty acid, being resinous and heavier than water.

The calcium salt was prepared and was found to contain 12·70 % Ca. Calcium caproate contains 14·81 % Ca, hence the non-volatile acid has a higher molecular weight than caproic acid.

Summary and Conclusions.

Calcium lactate, as was shown by Hoppe-Seyler, when fused with soda lime or caustic potash and magnesia, gives rise to a series of acids of the acetic series. In this Paper direct evidence of the formation of formic, acetic, propionic, butyric, and isobutyric acids has been given, but whether normal caproic acid is also formed is very doubtful.

The non-volatile acids of high molecular weight which are formed are not higher fatty acids, as stated by Hoppe-Seyler, since they are heavier than water, and since they are at least in part unsaturated, whilst their molecular weight is not far removed from that of caproic acid.

There is therefore no ground for stating that the acids having a molecular weight higher than that of propionic acid obtained by fusing calcium lactate with caustic alkalies, are 'characteristically normal,' as Hoppe-Seyler stated, for besides normal butyric acid, there is also formed isobutyric acid.

It follows also that the mode of decomposition of lactic acid when it is heated with alkalies cannot be used as an argument for the formation of fat from carbohydrates.

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UPON THE ACTIVE IMMUNISATION OF EXPERIMENTAL ANIMALS WITH TYPHOID CELL JUICES.

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THE active immunisation of experimental animals against the *B. typhosus* is readily accomplished, and the method is of a comparatively simple nature. If, for example, killed typhoid bacilli are injected subcutaneously, an immunity to otherwise fatal doses of the organism is established in the treated animal. The immunity thus acquired is characterised by the appearance in the blood of specific antibacterial substances. This is the essence of antityphoid inoculation as employed in the case of the human subject. The injection of suitable doses of killed typhoid bacilli evokes in the blood a condition similar to that occurring in experimental animals, and also to that occurring after recovery from a natural typhoid infection.

The vaccinating properties of dead typhoid cultures have been demonstrated notably by the work of Pfeiffer and Kolle,[1] and of Wright and Leishman.[2] The best means to be adopted for preparing and standardising the antityphoid vaccine is, however, a subject that admittedly demands further and careful study.

A conclusion, now generally accepted, is that the protective properties of the typhoid vaccine, however prepared, depend upon the bacilli contained therein. The bodies of the bacilli after injection become dissolved and their soluble immunising constituents are absorbed into the system. The immunising effect, under such conditions, is due to a utilisation of certain soluble intracellular elements of the typhoid bacillus. This being the case, it was natural that attempts should be made to obtain the immunising principles directly from the bacterial cell and to put the products to experimental tests. There are various ways in which this can be effected. A micro-organism may be dissolved with the aid of an alkali,[3] or submitted to a process of autolysis,[4] or the fresh plasma may be obtained by a mechanical trituration of the cell.[5] A discus-

sion of the relative merits of such methods of procedure is outside the scope of the present paper. The respective methods have as their common object the extraction of immunising substances from the bacterial cell, and in this particular instance from the typhoid bacillus. The purpose of the following enquiry was to test the feasibility of preparing a bacteria-free and fluid vaccine, and of standardising the same, *not in terms of bacilli, but in terms of the soluble immunising substances it might happen to contain.* The material was obtained with the aid of the cold-grinding method employed by Macfadyen and Rowland (*Centralblatt f. Bakteriologie*, vol. xxxiv., 1903). The typhoid organisms were grown on the surface of agar bottles at blood heat for about twenty hours. The growth was washed off with salt solution, and the bacteria spun out in a high speed centrifuge. The spun-out bacteria were next reduced to the consistency of a pasty mass on the surface of a Chamberland filter, and triturated at the temperature of liquid air. The resultant mass was then taken up in salt solution and centrifugalised. The cell juice was, as a rule, equivalent to a 10 per cent. solution of the intracellular constituents obtained. This material will in the following account be referred to as "typhoid cell juice," and contained from ·15 to ·2 per cent. of solid matter.

TABLE I.

Typhoid Cell Juice, 0·3 cc., was lethal within twenty-four hours to 200-250 grammes weight of guinea-pig on intraperitoneal injection.
Broth Culture B.T.A., 0·5 cc., was lethal in twenty-four hours for 250 grammes weight of guinea-pig on intraperitoneal injection.

Guinea-pigs' Weights, 320-400 Grammes.	Dose of Typhoid Cell Juice	Test Dose.	Day of Test	Alive after Twenty-four Hours	Day of Death reckoned from Inoculation of Cell Juice
1	0·4 cc.	0·8 cc.	7th	+	25th
2	0·4 ,,	0·8 ,,	,,	+	47th
3	0·2 ,,	0·8 ,,	,,	+	49th
4	0·2 ,,	0·8 ,,	,,	+	28th
5	0·1 ,,	0·8 ,,	,,	+	68th
6	0·1 ,,	0·8 ,,	,,	+	42nd
7	0·08 ,,	0·8 ,,	,,	+	49th
8	0·08 ,,	0·8 ,,	,,	+	53rd
9	0·06 ,,	0·8 ,,	,,	+	49th
10	0·06 ,,	0·8 ,,	,,	+	26th
11	0·04 ,,	0·7 ,,	,,	0	—
12	0·04 ,,	0·7 ,,	,,	+	45th
13	0·02 ,,	0·8 ,,	,,	+	Record lost.
14	0·02 ,,	0·8 ,,	,,	+	36th
Controls					
1	—	0·6 ,,	—	0	—
2	—	0·5 ,,	—	0	—

The material was, to begin with, injected subcutaneously into guinea-pigs, and after varying intervals of time, the protection afforded was tested by the intraperitoneal injection of virulent typhoid bacilli, *i.e.*, of cultures which killed the control animals within twenty-four hours. The same strain of *B. typhosus*, unless otherwise stated, was employed for the preparation of the typhoid cell juice, and for the cultures used as tests.

The details of the preliminary experiments are given in the subjoined tables, and it is to be noted that the dosage consisted of given amounts of the raw cell juice.

It will be seen that thirteen out of fourteen animals survived the test. These were kept under observation and after a time were noticed to lose condition, and eventually all died.

Post mortem.—The signs usually found were emaciation, congestion of the liver, and congestion of the lungs in this order of frequency. Cultures were made from the heart blood, the peritoneum and the spleen in most cases. From some of the animals no organisms were obtained, whilst from others the *B. typhosus*, at times in pure culture, was isolated. In this series, No. 14 gave *B. typhosus* after the longest interval, viz., twenty-nine days from the injection of the test dose, the organism being recovered from the peritoneal cavity.

TABLE II.

This experiment was a repetition of that given above.
Typhoid Cell Juice, 0·3 cc., lethal in twenty-four hours, for 250 grammes.
Broth Culture B.T.A., 0·1 cc. lethal in twenty-four hours, for 25 grammes.

Guinea-pigs' Weights	Dose of Typhoid Cell Juice	Test Dose	Day of Test	Alive after Twenty-four Hours	Day of Death reckoned from Inoculation of Cell Juice
430 grms.	0·4 cc.	1·7 cc.	7th	+	63rd
460 ,,	0·4 ,,	1·8 ,,	,,	+	48th
440 ,,	0·2 ,,	1·7 ,,	,,	+	57th
370 ,,	0·2 ,,	1·5 ,,	,,	+	38th
320 ,,	0·1 ,,	1·3 ,,	,,	+	53rd
340 ,,	0·1 ,,	1·4 ,,	,,	+	55th
290 ,,	0·08 ,,	1·2 ,,	,,	0	—
300 ,,	0·08 ,,	1·2 ,,	,,	+	16th
290 ,,	0·06 ,,	1·2 ,,	,,	+	66th
300 ,,	0·06 ,,	1·2 ,,	,,	+	72nd
270 ,,	0·04 ,,	1·1 ,,	,,	0	—
250 ,,	0·04 ,,	1·0 ,,	,,	+	41st
250 ,,	0·02 ,,	1·0 ,,	,,	+	10th
260 ,,	0·02 ,,	1·0 ,,	,,	+	24th
Controls					
220 ,,	—	0·9 ,,	—	0	—
310 ,,	—	1·3 ,,	—	0	—

The results in this case were very similar to those of the first series—twelve of the vaccinated animals survived out of fourteen tested. The same loss of condition was observed, and all ultimately died after about the same interval of time. On cultures being made, in some cases no organisms were recovered, in other cases the *B. typhosus* was detected—in one instance thirty-four days after the injection of the test dose, and again from the peritoneal cavity.

It having been demonstrated by the preceding experiments that the subcutaneous injection of typhoid cell juice in varying doses enabled the animals at the end of a week to withstand for a variable time (ten to seventy-two days) a dose of living organisms sufficient to kill the control animals within twenty-four hours, it appeared desirable to endeavour to find out how long this immunity lasted and in how far it was dependent on the amount of material injected. For this purpose a large series of animals were taken and received subcutaneous injections of the typhoid cell juice in doses varying from $\frac{1}{10}$ to $\frac{1}{50}$ cc.

TABLE III.

Typhoid Cell Juice, 0·2 cc. to 0·3 cc., lethal in twenty-four hours for 200-250 grammes. *Broth Culture B.T.A.*, lethal dose was variable.

Guinea-pigs' Weights	Dose of Typhoid Cell Juice	Test Dose	Day of Test	Alive after Twenty-four Hours	Day of Death, reckoned from Inoculation of T. Cell Juice
270 grms.	0·1 cc.	0·5 cc.	14th	+	63rd
220 ,,	0·08 ,,	0·3 ,,	,,	+	30th
200 ,,	0·06 ,,	0·3 ,,	,,	+	28th
270 ,,	0·04 ,,	0·4 ,,	,,	+	49th
230 ,,	0·02 ,,	0·4 ,,	,,	0	—
210 ,,	0·02 ,,	0·4 ,,	15th	+	28th
210 ,,	0·02 ,,	0·4 ,,	,,	0	—
230 ,,	0·04 ,,	0·4 ,,	21st	+	31st
230 ,,	0·02 ,,	0·4 ,,	,,	+	24th
Controls					
180 ,,	—	0·3 ,,	14th	0	—
180 ,,	—	0·3 ,,	,,	+	—
160 ,,	—	0·2 ,,	15th	+	—
270 ,,	—	0·4 ,,	21st	0	—

Of nine animals tested on the 14th, 15th and 21st day, seven survived. At this point it was realised that the experiment would fail to give the information desired, as it was found that out of the remaining (untested) animals, twenty-eight in number, no less than eight were dead on the 51st day after inoculation. This question of late death will be referred to below in more detail. The positive

results showed so far evidence of protection at the end of three weeks.

The next Table gives the tests made upon those animals which were still alive forty-two days after inoculation.

TABLE IV.

Eight more were tested on the forty-second day.

The animal marked * was noted to be ill at the time of testing, so may perhaps be excluded.

Guinea-pigs' Weights	Dose of Typhoid Cell Juice	Test Dose	Day of Test	Alive after Twenty-four Hours	Day of Death, reckoned from Inoculation of T. Cell Juice
410 grms.	0·1 cc.	1·0 cc.	42nd	0	—
250 ,,	0·1 ,,	0·5 ,,	,,	0	—
280 ,,	0·25 ,,	0·5 ,,	,,	+	59th
*210 ,,	0·5 ,,	0·5 ,,	,,	0	—
330 ,,	0·75 ,,	1·0 ,,	,,	+	43rd
420 ,,	0·75 ,,	1·0 ,,	,,	+	Record lost.
260 ,,	1·0 ,,	0·5 ,,	,,	+	60th
250 ,,	1·0 ,,	0·5 ,,	,,	+	45th
Controls					
420 ,,	—	1·0 ,,	—	0	—
210 ,,	—	0·5 ,,	—	0	—

In the above experiment there was also evidence of protection at the end of six weeks.

The Table that follows is of interest as showing the effect of the chronic intoxication resulting from the injection of varying doses of typhoid cell juice only.

TABLE V.

Guinea-pigs' Weights	Dose of Typhoid Cell Juice	Day of Death	Results of Cultures made
210 grms.	0·04 cc.	21st	Sterile.
200 ,,	0·06 ,,	,,	,,
230 ,,	0·06 ,,	,,	,,
220 ,,	0·08 ,,	,,	B.T.A.
210 ,,	0·08 ,,	36th	Sterile.
210 ,,	0·08 ,,	21st	,,
270 ,,	0·1 ,,	28th	,,
200 ,,	0·25 ,,	,,	,,
240 ,,	0·25 ,,	25th	,,
250 ,,	0·25 ,,	28th	,,
270 ,,	0·5 ,,	42nd	,,
270 ,,	0·5 ,,	21st	,,
250 ,,	0·5 ,,	,,	,,
220 ,,	0·5 ,,	,,	,,
250 ,,	0·75 ,,	28th	,,
300 ,,	0·75 ,,	31st	,,
250 ,,	1·0 ,,	28th	,,
250 ,,	1·0 ,,	21st	,,

The point brought into prominence in this experiment was that the typhoid cell juice *per se* produced a toxic effect upon the animals, which first showed itself about the 21st day, by the occurrence of a large proportion of deaths among those remaining untested. *Post mortem* all the animals showed emaciation, some congestion of the liver, a smaller number congestion of the lungs, while in others no naked-eye signs sufficient to account for death could be seen. It is probable that those animals in Tables I. and II., which, after treatment with typhoid cell juice and testing with living organisms, died and proved sterile on culture, were suffering from the effect of the same toxic agent contained in the material. It is also possible that the onset of these effects caused the irregularity of the results in Table III., which were noticeable after the 14th day. In the course of another experiment, six guinea-pigs, which had received $\frac{1}{50}$ cc., of the material subcutaneously, died at intervals of from fourteen to thirty-seven days, and all proved sterile on culture.

That the toxic substance exists in the bodies of the bacilli is indicated by the following experiment. The same virulent strain of organism from which the cell juices in the preceding cases was prepared was cultivated for twenty-four hours on agar in Roux flasks. The growth was washed off in 100 cc. sterile normal salt solution, and then sterilised by heating at 60° C. for fifteen minutes. The resulting suspension was lethal in a dose of 0·5 cc. to a guinea-pig of 200 grammes' weight on intraperitoneal injection. This suspension was injected subcutaneously, as shown below.

Doses ..	0·8 cc.	0·4 cc.	0·2 cc.	0·08 cc.	0·04 cc.	0·02 cc.
Weights of guinea-pigs	220 grms. 220 ,,	260 grms. 220 ,,	210 grms. 220 ,,	230 grms. 220 ,,	240 grms. 230 ,,	200 grms. 230 ,,

The animals died with emaciation at intervals of fourteen to forty-two days after injection, and all proved sterile on culture with one exception, from which a non-typhoid organism was isolated. The sharp limitation of the interval between injection and death, both in the case of the typhoid cell juice and in that of the killed organisms, is noticeable, and the commonest time of death was at the end of three weeks, several animals succumbing on the 21st day. It has not been demonstrated that as regards toxicity any marked differences exist between killed individual strains of the *B. typhosus*.

Typhoid cell juices which had been treated with chloroform vapour and were non-lethal on intraperitoneal injection of 0·5

cc., produced a similar intoxication subcutaneously. A further observation, though not in chronological order, may be quoted here. Six guinea-pigs (200—225 grammes) were inoculated, three with ·001, and three with ·0001 cc. of the cell juice. Four were dead at the end of forty-nine days, viz., two out of each group, despite the very small dose employed. On another occasion a guinea-pig weighing 750 grammes received 0·5 cc. of a typhoid cell juice which had been passed through a Berkefeld filter. The animal likewise died with the usual signs on the 40th day. There exists therefore in the bodies and in the fluid extracts of typhoid bacilli, a substance lethal to guinea-pigs at the end of about three weeks, and it is likewise present in filtered juices.

The foregoing results have been given somewhat in detail, as they appear to be of practical significance and to raise a question of considerable importance in connection with typhoid, and possibly other allied vaccines, inasmuch as if the observed toxicity is a negligible factor in the immunising process, its abolition or reduction to a minimum would be not a loss but a gain.

In the above experiments the broad fact emerged that following the doses of cell juices or killed bacilli employed, the vaccinated animals, whilst developing a certain degree of immunity, suffered concurrently from an intoxication, and that this complication ultimately resulted in death. The protective forces of the animals were not sufficient to overcome this intoxication. The guinea-pig proved markedly susceptible, so that it was impossible to determine the duration of the vaccinating effect after a certain period of time, whilst it was a complicating factor to be eliminated, if possible, in subsequent experiments with this particular species of animal. In view of this disturbing effect occurring in doses as small as $\frac{1}{50}$ cc., the next step was to enquire whether still smaller doses might not protect the animals as efficiently, or even more so, by doing away with or lessening the intoxication.

Eighteen guinea-pigs were inoculated with doses ranging from ·06 down to ·006 cc. of typhoid cell juice. The only fresh point that emerged was that ·006 cc. had the same protective effect as the larger doses, and that after a period of eleven days. The chronic intoxication already described still supervened. It appeared possible that the lowest effective dose had not been reached and the process was carried further. The Table following gives the results.

A protective effect was demonstrable with a dose of $\frac{1}{1000}$ cc., at the end of seven days. A corroborative experiment was made to test the efficacy of $\frac{1}{1000}$ cc. of the material as a protec-

tive inoculation. The animals were tested on the 13th day after inoculation and survived two lethal doses of the *B. typhosus*. An endeavour was also made to determine on what day after an inoculation the protection afforded by it was established. The experiments were few in number, but they indicated that protection appears to be established about the 7th day.

TABLE VI.

Typhoid Cell Juice, lethal in dose of 0·3 cc. for 200–250 grammes in twenty-four hours. *Broth Culture B.T.A.*, virulence variable.

Guinea-pigs' Weights	Dose of Typhoid Cell Juice	Test Dose	Day of Test	Alive after Twenty-four Hours	Day of Death reckoned from Inoculation of Cell Juice
220 grms.	0·01 cc.	0·5 cc.	7th	+	44th
220 ,,	0·008 ,,	0·5 ,,	,,	+	11th
210 ,,	0·006 ,,	0·5 ,,	,,	+	30th
200 ,,	0·004 ,,	0·5 ,,	,,	+	24th
200 ,,	0·002 ,,	0·5 ,,	,,	+	33rd
190 ,,	0·001 ,,	0·5 ,,	,,	+	45th
220 ,,	0·01 ,,	0·5 ,,	8th	+	29th
220 ,,	0·008 ,,	0·5 ,,	,,	+	22nd
210 ,,	0·006 ,,	0·5 ,,	,,	+	33rd
200 ,,	0·004 ,,	0·5 ,,	,,	+	16th
200 ,,	0 002 ,,	0·5 ,,	,,	+	30th
180 ,,	0·001 ,,	0·5 ,,	,,	+	26th
230 ,,	0·01 ,,	0·75 ,,	9th	0	—
210 ,,	0·008 ,,	0·5 ,,	,,	+	12th
200 ,,	0·006 ,,	0·5 ,,	,,	+	30th
200 ,,	0·004 ,,	0·75 ,,	,,	+	30th
190 ,,	0·002 ,,	0·75 ,,	,,	+	54th
180 ,,	0·001 ,,	0·5 ,,	,,	0	—
Controls.					
200 grms.	—	0·5 cc.	7th	0	—
200 ,,	—	0·5 ,,	,,	0	—
170 ,,		0·5 ,,	8th	+	—
170 ,,	—	0·5 ,,	,,	0	—
120 ,,	—	0·5 ,,	9th	0	—
320 ,,	—	0·75 ,,	,,	0	—

A series of animals which had been inoculated with 0·25 cc. of the cell juice were tested on the 10th day after injection; out of five guinea-pigs tested four survived an injection of 3 to 5 lethal doses of the *B. typhosus*.

The above experiments had so far demonstrated: (1) The practicability of extracting immunising substances from the typhoid bacillus; (2) the occurrence of a chronic intoxication which acted as a disturbing factor in the immunising process.

The next series of experiments was conducted with a view to determining the presence of agglutinating and bactericidal substances in the blood of animals after injection of the typhoid cell

juice. Rabbits were chiefly employed for this purpose, and the smallest dose which had proved effectual in the guinea-pig was injected intravenously. The bactericidal power of the various sera was estimated in the manner described by Wright (*Proc. Royal Society*, vol. lxxi., 1902), usually at an interval of three to four hours after the collection of the blood. The agglutinins were determined by hanging drop preparations observed at the end of one hour, the arbitrary end point being "clumping" with loss of motility in any organisms that were lying free.

Experiment I.

A cell juice from a typhoid strain of low virulence was employed, *i.e.*, from an organism which had been cultivated in the laboratory for at least twelve months, and of which the lethal dose was 1 cc. of a broth culture. Of this sterile cell juice, $\frac{1}{1000}$ cc. (·00002 gramme), was injected intravenously in two rabbits. The serum

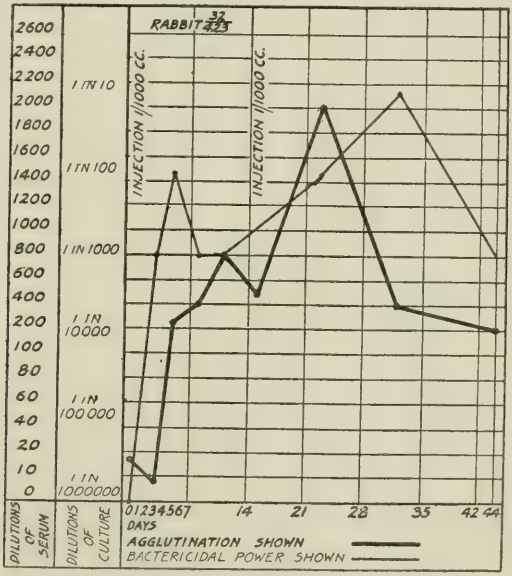

Chart I.

estimations were carried out with the same strain of bacillus. One of the rabbits died on the 7th day after inoculation from right-sided pleuro-pneumonia. In both rabbits the agglutinating value of the serum was lower on the 2nd day after inoculation than it was before treatment, while the bactericidal power was already raised. In Chart I. the agglutination and bactericidal curves of the surviving

rabbit are given after two injections of $\frac{1}{1000}$ cc., the second injection having been given fourteen days later.

Experiment II.

The cell juice employed in the first experiment was evaporated to dryness and taken up in a 1 per cent. solution of sodic carbonate in the proportion of 1 mgr. to 1 cc. of the solvent. Two rabbits received 1 cc. of this solution (·001 gramme) intravenously. The

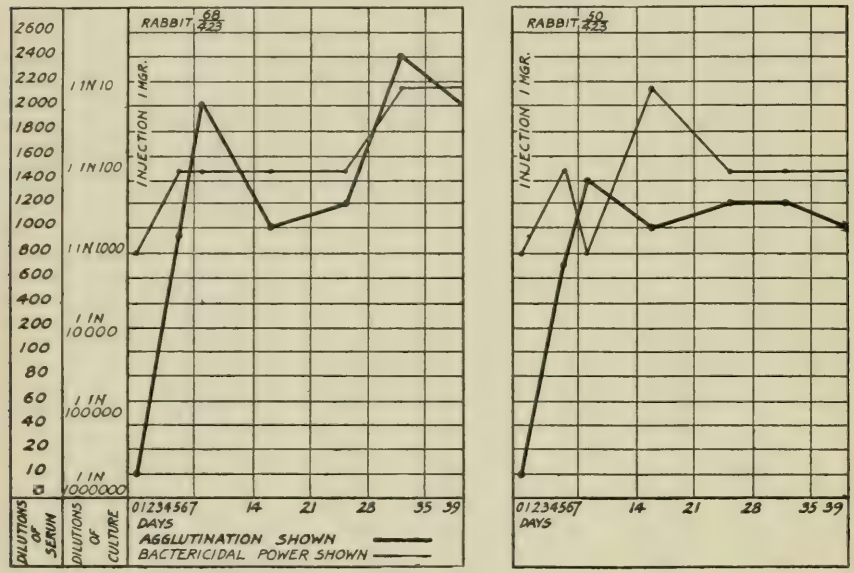

Chart II.

interest of this experiment lay in the ability to show that after reducing the material to a dried form, the residue may be taken up in a suitable fluid, and will on injection cause the appearance of specific immunising substances in the blood. The results are given in Chart II. The bactericidal power of the blood prior to inoculation stood at rather a high level, but a rise was manifest four days after injection. It remained at this higher level in the case of the first rabbit for three weeks, during which time three further estimations were made. There was a further rise maintained for at least a week. The second animal shows more and greater fluctations, but in both cases there was at the end of thirty-nine days a higher bactericidal value than existed prior to inoculation. A fall in the agglutination curve occurred in both cases from the 32nd to the 39th day.

Experiment III.

The typhoid cell juice was prepared from a strain of high virulence, and was passed through a Berkefeld filter. The filtrate was evaporated to dryness, and taken up in sodic carbonate solution in the proportion of 1 mgr. to 1 cc. of the solvent. Two rabbits received respectively 1 and 0·8 cc. intravenously. The animal which had received the larger dose died at the end of a fortnight. Chart III. shows the curves of the surviving rabbit up to the end of seventy-one days. There was in this instance also a delayed rise in the agglutinating power of the blood, but an immediate rise in the bactericidal power. It will be seen that seventy days after a single injection of the material an increased agglutinating and bactericidal power was present in the blood serum as compared with that existing in it prior to the inoculation.

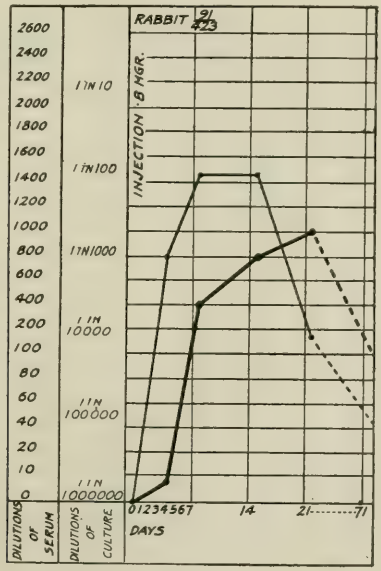

Chart III.

An experiment made on a *Rhesus* monkey gave rise to a similar result on injecting 0·5 cc. of the typhoid cell juice subcutaneously. At the end of fourteen days the bactericidal power of the monkey's blood had risen from 1 : 10,000 (negative) to 1 : 10 (positive), as tested against a virulent strain of the *B. typhosus.*

The experiments at this stage had adequately shown that the unfiltered and also the filtered typhoid cell juice gave rise to a

distinct production of agglutinating and bactericidal substances in the serum of the treated animals, and that this effect could be produced by an injection of $\frac{1}{1000}$ cc. of the cell juice, and by 1 mgr. of its dried residue.

The concluding experiments were directed to placing the results on a more exact quantitative basis, and were carried out with filtered juices containing only soluble constituents of the typhoid bacillus and free from organisms. The protective value of the inoculations was tested directly on guinea-pigs of an average weight of 300—350 grammes against multiple lethal doses of the *B. typhosus*. The same virulent strain of organism was employed for the preparation of the cell juices and for the subsequent tests.

The following Table gives the results of the subcutaneous inoculation of a typhoid cell juice before and after filtration.

SUBCUTANEOUS INOCULATION OF UNFILTERED AND FILTERED TYPHOID CELL JUICE.

Guinea-pigs. 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	·02 cc. *unfiltered*	10 lethal doses	7th	+	—
2	·02 ,, ,,	5 ,,	,,	+	—
3	·01 ,, ,,	10 ,,	,,	0	Died before test.
4	·01 ,, ,,	5 ,,	,,	+	—
5	·005 ,, ,,	10 ,,	,,	+	—
6	·005 ,, ,,	5 ,,	,,	+	—
1	·02 ,, *filtered*	10 ,,	,,	+	—
2	·02 ,, ,,	5 ,,	,,	+	—
3	·01 ,, ,,	10 ,,	,,	+	—
4	·01 ,, ,,	5 ,,	,,	+	—
5	·005 ,, ,,	10 ,,	,,	0	—
6	·005 ,, ,,	5 ,,	,,	+	—

In the doses employed the filtered juice approximated very closely in protective power to the unfiltered juice. The only animals showing signs of local infiltration were those which had received the two highest doses of the unfiltered material.

The five series of experiments detailed below were made with filtered typhoid cell juices. The amount of solid matter present in 1 cc. of the filtered juice was in each instance estimated. This was done by drying a measured quantity of the juice *in vacuo* over sulphuric acid, and weighing the residue. It was therefore possible to gauge the amount of soluble matter contained in the respective fluid doses employed. In Series III., IV. and V., the filtered juice was diluted so that 1 cc. corresponded to 1 mgr. of solid matter. The further particulars will be found in the Tables.

Subcutaneous Inoculation of Filtered Typhoid Cell Juice.

Series I.

1 cc. contained 3½ milligrammes solids.

Guinea-pigs 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	1 cc.	8-10 lethal doses	7th	+	—
2	1 ,,	,, ,,	,,	0	—
3	0·5 ,,	,, ,,	,,	+	—
4	0·5 ,,	,, ,,	,,	+	—
5	0·2 ,,	,, ,,	,,	+	—
6	0·2 ,,	,, ,,	,,	+	—
7	0·1 ,,	,, ,,	,,	0	Died before test.
8	0·1 ,,	,, ,,	,,	+	—
9	0·05 ,,	,, ,,	,,	+	—
10	0·05 ,,	,, ,,	,,	0	—

Series II.

1 cc. contained 1·2 milligrammes solids.

Guinea-pigs 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	1 cc.	8-10 lethal doses	7th	+	—
2	1 ,,	,, ,,	,,	0	—
3	0·5 ,,	,, ,,	,,	+	—
4	0·5 ,,	,, ,,	,,	+	—
5	0·3 ,,	,, ,,	,,	+	—
6	0·3 ,,	,, ,,	,,	+	—
7	0·2 ,,	,, ,,	,,	+	—
8	0·2 ,,	,, ,,	,,	+	—
9	0·1 ,,	,, ,,	,,	+	—
10	0·1 ,,	,, ,,	,,	0	Died before test.

Series III.

1 cc. contained 1 milligramme solids.

Guinea-pigs, 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	2 cc.	8-10 lethal doses	11th	+	—
2	2 ,,	,, ,,	,,	+	—
3	1 ,,	,, ,,	,,	+	—
4	1 ,,	,, ,,	,,	0	Nodular spleen.
5	0·5 ,,	,, ,,	,,	0	,, ,,
6	0·5 ,,	,, ,,	,,	+	—
7	0·3 ,,	,, ,,	,,	0	Nodular spleen.
8	0·3 ,,	,, ,,	,,	+	—
9	0·2 ,,	,, ,,	,,	+	—
10	0·2 ,,	,, ,,	,,	+	—
11	0·1 ,,	,, ,,	,,	+	—
12	0·1 ,,	,, ,,	,,	+	—

Series IV.

1 cc. contained 1 milligramme solids.

Guinea-pigs. 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	2 cc.	8-10 lethal doses	8th	+	—
2	2 ,,	,, ,,	,,	+	—
3	1 ,,	,, ,,	,,	0	—
4	1 ,,	,, ,,	,,	0	Nodular spleen.
5	0·5 ,,	,, ,,	,,	+	—
6	0·5 ,,	,, ,,	,,	+	—
7	0·3 ,,	,, ,,	,,	0	Died before test.
8	0·3 ,,	,, ,,	,,	+	—
9	0·2 ,,	,, ,,	,,	+	—
10	0·2 ,,	,, ,,	,,	0	Nodular spleen.
11	0·1 ,,	,, ,,	,,	+	—
12	0·1 ,,	,, ,,	,,	+	—

Series V.

1 cc. contained 1 milligramme solids.

Guinea-pigs. 300-350 grms.	Dose of Juice	Test Dose of *B. typhosus*	Day of Test	Alive after Twenty-four Hours	Remarks
1	1 cc.	8-10 lethal doses	8th	+	—
2	1 ,,	,, ,,	,,	+	—
3	0·5 ,,	,, ,,	,,	+	—
4	0·5 ,,	,, ,,	,,	+	—
5	0·1 ,,	,, ,,	,,	+	—
6	0·1 ,,	,, ,,	,,	+	—
1	1 ,,	,, ,,	,,	+	—
2	1 ,,	,, ,,	,,	+	—
3	0·5 ,,	,, ,,	,,	+	—
4	0·5 ,,	,, ,,	,,	+	—
5	0·1 ,,	,, ,,	,,	+	—
6	0·1 ,,	,, ,,	,,	+	—

It is perhaps hardly necessary to comment upon these results. There was again complete absorption, and an absence of infiltration at the seat of inoculation. The filtered juices in doses corresponding to 1 mgr. of solid matter, protected the animals when tested on the 8th and 11th day against 8 to 10 lethal doses of the typhoid bacillus injected into the peritoneal cavity. A control of the number of lethal doses injected was carried out in each instance. The majority of the animals that succumbed after injection of the test doses were found to be in a diseased condition, and were consequently hypersensitive. Whilst the smallest effective dose of the filtered material in these experiments proved to be about $\frac{1}{10}$ mgr., it is possible that if still smaller amounts had been used an equivalent protective effect would have resulted.

The last series of experiments was made on the rabbit. The filtered typhoid cell juice was injected intravenously in doses that varied from about 1 to $\frac{1}{100}$ mgr. of solid matter. The protective power of the rabbits' serum was tested *in vivo* on the guinea-pig. The serum dilutions were mixed with 8 to 10 lethal doses of the *B. typhosus* and the mixture was injected into the peritoneal cavity of the guinea-pig, controls being made at the same time. The amount of serum capable of saving the life of the guinea-pig was noted and is recorded in the Table. The tests were not carried further than with $\frac{1}{200}$ cc. of the serum of the treated rabbits. The experiments, therefore, simply record the amount of soluble cell juice capable of raising the bacteriolytic titrate of the serum to $\frac{1}{200}$ cc. The possibility of still smaller doses being equally effective is not, of course, excluded. It was previously established that ·5 cc. of the normal rabbits' serum did not protect a guinea-pig against 8 to 10 lethal doses of the typhoid organism. The agglutinins were not particularly considered, greater value being attached to the actual protective power developed in the serum of the animals against an otherwise fatal infection with the *B. typhosus.*

INTRAVENOUS INJECTION OF TYPHOID CELL JUICE IN RABBITS (2,000 TO 2,500 GRAMMES WEIGHT).

Rabbit	Dose in Milligrammes of Solids	Test Dose of *B. typhosus*	Day of Test	Agglutinin Titrate of Serum	Bacteriolytic Titrate of Serum
	1	8-10 lethal doses	8th	1 : 1,000 = +	·005 cc.
	$\frac{3}{10}$	,, ,,	8th	1 : 1,000 = +	·005 ,,
	$\frac{5}{10}$	,, ,,	13th	1 : 1,000 = +	·005 ,,
	$\frac{5}{10}$	,, ,,	13th	1 : 1,000 = +	·005 ,,
	1	,, ,,	8th	1 : 1,000 = +	·005 ,,
	$\frac{1}{2}$	,, ,,	8th	1 : 1,000 = +	·005 ,,
	$\frac{1}{10}$	,, ,,	8th	1 : 1,000 = +	·005 ,,
	$\frac{1}{1000}$	,, ,,	8th	1 : 1,000 = +	·005 ,,

The results show that following the injection of the filtered fluids there occurred a distinct production of agglutinins and bacteriolysins in the blood of the treated animals. The serum in an amount of $\frac{1}{200}$ cc. protected guinea-pigs against 8 to 10 lethal doses of the *B. typhosus.* It will be seen that within the limits of the experiments about $\frac{1}{100}$ part of a mgr. produced an effect equivalent to that following the injection of 1 mgr. of the soluble material. This difference is a striking one, and would appear to indicate that the immunising substances, when extracted from

the typhoid bacillus in a soluble form, are demonstrably effective in very small amount. That the smallest effective dose had possibly not been reached was suggested by an experiment in which the intravenous injection of $\frac{1}{1000}$ cc. of a filtered juice resulted in a positive agglutination reaction with the rabbits' serum in a dilution of $\frac{1}{1000}$.

Conclusions.

(1) A bacteria-free and fluid typhoid vaccine can be prepared by the mechanical method employed.

(2) The fluid vaccine can be standardised in terms of the soluble immunising substances it may happen to contain.

(3) A very small amount of the soluble constituents of the typhoid organism is necessary for the active immunisation of experimental animals.

It is with much pleasure that I take this opportunity of expressing my gratitude to Dr. Allan Macfadyen, at whose suggestion I undertook the above investigation. Dr. Macfayden has not only assisted me throughout the work with advice and criticism, but as I was obliged to leave for foreign service before its completion, has had the great kindness to complete the observations by a large number of further experiments, and has also been put to the trouble of preparing the paper in a form suitable for publication.

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ON THE OCCURRENCE OF THE MICROCOCCUS CATARRHALIS IN NORMAL AND CATARRHAL NOSES AND ITS DIFFERENTIATION FROM OTHER GRAM-NEGATIVE COCCI.

By J. A. ARKWRIGHT, M.D.

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The *Micrococcus catarrhalis* was first isolated by Kirchner (1890) from cases of influenza-like illness. Since then several epidemics of acute catarrh and bronchitis associated with this organism have been recorded.

The first epidemic recorded in this country was that described by Dunn and Gordon (1905) as occurring in Hertfordshire; the clinical aspect of the disease was that of an acute febrile illness with variable symptoms, of which sore throat, scarlatiniform eruptions, and symptoms suggesting meningitis were the most striking features. Other epidemics have been recorded by Ghon and Pfeiffer (1902), Bezançon and de Jong (1905), and others.

In a large proportion of the cases examined Gordon obtained the *Micrococcus catarrhalis* in great numbers and often in pure culture from the nose and throat. Several writers have described the occurrence of other Gram-negative cocci in the nose.

The Meningococcus (or *Diplococcus intracellularis meningitidis* of Weichselbaum) has been found in the naso-pharynx and nasal fossae, almost exclusively in cases of meningitis or in those in close contact with meningitis patients. A few occurrences have been, however, recorded apart from obvious meningitis infection by Schiff (1898), Weichselbaum and Ghon (1905), and others.

Dunham (1906) examined cultures from the nose and throat of 16 persons suffering from epidemic meningitis, and in six found an organism resembling the meningococcus in every respect, including agglutination. Goodwin and Sholly (1906) examined the noses of

55 healthy persons not in contact with meningitis cases, and on two occasions isolated an organism which resembled the meningococcus in its cultural characteristics, but differed from it in its agglutinating properties. They also examined the noses of 45 persons in close contact with meningitis cases and found the meningococcus in five, *i.e.* 11 per cent.

v. Lingelsheim (1906) examined the naso-pharyngeal secretion of 346 persons who were in health, or ill of some complaint other than meningitis, and who were not in contact with meningitis cases: in none did he find the meningococcus. In 125 persons in more or less close contact with meningitis cases he found the meningococcus in 24 (= 19 %). On the other hand, he examined the nasal secretion in 787 cases of clinical meningitis and found the meningococcus in 182 (= 25 %). In 49 cases examined carefully in hospital in the first five days of their illness 46 (= 93·8 %) yielded the meningococcus.

Kutscher (1906) in Berlin examined the noses of 56 patients suffering from other complaints, and two of these yielded organisms which resembled the meningococcus in every respect, including agglutination; two yielded cocci like the meningococcus, but the agglutination test was not applied as the cultures died out. According to Kolle and Wassermann (1906), 114 persons were examined in Berlin with similar results.

In the identification of *Micrococcus catarrhalis* the most important organisms that require to be considered are the meningococcus, gonococcus, and certain other races of Gram-negative cocci, which occur in the nose and elsewhere, but have not yet been clearly differentiated.

The three first-mentioned organisms resemble each other very closely in several particulars.

1. In the body the cocci are often found exclusively inside the polymorphonuclear leucocytes.

2. When first removed from the body they have very feeble powers of growing on artificial media, usually requiring the addition of blood, or ascitic fluid or serum to the medium.

3. They do not usually survive long without sub-culture.

4. They are very easily killed, especially when first isolated, by drying.

5. They are very feebly, or often not at all, pathogenic for laboratory animals.

6. Morphologically there is a tendency for the cocci to vary very much in size on artificial media, especially after a few days' growth.

Individuals then occur which are two or three times the size of the ordinary forms, stain more deeply by methylene blue, and are not so readily decolourised by Gram's method.

7. They grow in pairs or tetrads, never in chains.

8. They all, when taken from the body or from a 24 hours' culture, decolourise rapidly and completely by Gram's method, except the giant forms mentioned above, which decolourise less rapidly.

The chief recognised points of difference between the several species are the following, and here I shall make use of the description by Neisser (1903) in Kolle and Wassermann's handbook unless another author is mentioned.

1. On agar with or without ascitic fluid the colonies of *M. catarrhalis* are thicker, more raised and more opaque than those of the meningococcus or gonococcus, and they appear coarsely granular under a low power of the microscope; they readily become confluent, and are of a firm consistency.

The colonies of the meningococcus, on the other hand, are soft and sticky, and appear smooth or only finely granular with low magnification. The colonies are often confluent when crowded.

Dunham (1906) considered that the mucilaginous nature of colonies of the meningococcus and some similar organisms was useful as a means of differentiating the various groups, as he found that this property caused an emulsion of these organisms to pass very slowly through filter paper.

2. Ghon and Pfeiffer (1902) say that their cultures of *M. catarrhalis* in *broth* usually formed a skin on the surface, and later a ring at the upper level of the liquid and a deposit, the broth remaining clear, but Neisser (1903) states that the broth becomes turbid and a deposit forms. Bezançon and de Jong (1905) observed a turbidity and powdery deposit.

The meningococcus causes general turbidity, and if the tube is kept very still a surface skin is said to form; whilst the gonococcus on the addition of ascitic fluid or serum to the broth grows as a coarsely granular surface skin and deposit leaving the broth clear.

3. When media containing various carbohydrates are used for cultures, the different groups do not produce acids from the same sugars. Dunn and Gordon (1905) noted the reactions in broth containing glucose, maltose, saccharose or galactose after incubation at 37° C. for seven days. He found that *M. catarrhalis* produced acid in none of these, and that the meningococcus did so in glucose,

maltose and galactose, and the gonococcus in glucose and galactose (when a few drops of ascitic fluid had been added). F. W. Andrewes (1906) adds dextrin and laevulose to those carbohydrates from which acid is formed by the meningococcus.

Dunham (1906), using litmus-glucose-serum-broth found that the meningococcus produced acid but no coagulation, and that catarrhalis-like organisms either caused no acidity or produced acid and also caused coagulation of the medium, whereas *M. catarrhalis* caused neither acidity nor coagulation.

4. Specific agglutination as a means of differentiation has proved of value as regards several kinds of Gram-negative organisms, but *M. catarrhalis* and some of the other species agglutinate spontaneously. Dunham (1906), Goodman and Sholly (1906), v. Lingelsheim (1906), and others have prepared meningococcus sera and have successfully used their agglutinating action to separate organisms otherwise indistinguishable from the meningococcus. v. Lingelsheim also prepared a *M. catarrhalis* serum which did not agglutinate the meningococcus.

Several other kinds of Gram-negative cocci have been isolated from the nose which do not conform to the characters of *M. catarrhalis*, meningococcus or gonococcus. Some of these are said to resemble catarrhalis in forming no acid in those sugars in which they were tested, others resemble catarrhalis in most respects, but produce acid in glucose, galactose, maltose and laevulose, and also in cane sugar, as pointed out by Dunn and Gordon (1905), who found as well another race which produced acid only in glucose and maltose.

v. Lingelsheim (1906) isolated five kinds of Gram-negative cocci from the naso-pharynx and differentiated them by the size and appearance of their colonies, by the production of yellow pigment, by the changes which they produced in solid media containing various carbohydrates and coloured with litmus, and by their agglutination reactions with sera prepared by injecting the meningococcus, catarrhalis or one of two other races which he had isolated.

I have taken the following as the chief differentiating characteristics of the principal Gram-negative cocci here considered:

M. catarrhalis. Growth on ordinary agar after the first isolation in translucent colonies which are whitish by reflected, and brown by transmitted light, and are seen to be coarsely granular with low magnification.

Growth fairly good on gelatin at 20° C.: inability to produce acid from any of the carbohydrates on which it has been tried.

Meningococcus. A less free growth on agar, the colonies translucent and slightly milky by reflected light; the appearance of the colonies with low magnification smooth or only finely granular; general turbidity in broth; the production of acid from maltose and usually from glucose, galactose and laevulose, but not from cane sugar. Absence of growth at 20° C. Agglutination with a serum prepared by injecting a rabbit with the meningococcus.

Gonococcus. Inability to grow on media without addition of serum or ascitic fluid, at least till several generations of artificial culture have been passed. Colonies very translucent, and not confluent. Inability to form acid in maltose or saccharose although glucose or galactose may be thus affected.

I have examined the nose in 54 instances by means of small swabs, which were introduced where possible through a speculum, and then smeared on the surface of a mixture of blood and agar, or of ascitic fluid, nutrose and agar, as recommended by Dunn and Gordon. In 19 instances I isolated *M. catarrhalis*, but from a much larger proportion of infants under one year than of older children or of adults: the last furnished proportionately the fewest strains. The proportion in catarrhal noses was no higher than in the normal noses. In only one case, a girl of 16 years of age, was *M. catarrhalis* obtained in pure culture from a nose, and this was on the fourth day of a case of acute nasal catarrh without much constitutional disturbance, when the catarrh was nearly at its height.

TABLE I. *Organisms isolated from* 53 *noses.*

	15 Normal	7 Discharging Post S. F.	26 Acutely catarrhal	5 Meningitis cases
M. catarrhalis	5	3	8	3
Percentage of cases examined in which *M. catarrhalis* was isolated	33	43	31	60
Pneumococcus	4	1	9	3
Percentage of cases examined	26	14	35	60
Hoffman's bacillus	2	4	11	—
Percentage of cases examined	13	49	42	—
Catarrhalis-like organisms	1	1	1	—

TABLE II.

Age:—	Under 1 year	1—14 years	Over 14 years	All ages
Number of noses examined ...	12	23	19	54
Number in which *M. catarrhalis* was found	7	8	4	19
Percentage with *M. catarrhalis* of those examined at each age	58	35	21	35

I examined the nose in five cases of meningitis, but not within the first five days of the illness. Many organisms appeared in the cultures, but in no instance did I find an organism resembling the meningococcus.

In order to compare the characteristics of *M. catarrhalis* with other cocci which resemble it morphologically and in their staining reactions, I have cultivated the following varieties of Gram-negative cocci.

1. Six strains of meningococcus.

2. *Strain* 4*M* isolated by Dr Marshall from fluid obtained by lumbar puncture in a case of meningitis supposed to be of tubercular origin.

3. *Gonococcus,* isolated by myself on blood agar from an acute case of gonorrhoea.

4. Nineteen strains of *M. catarrhalis,* which were all isolated from the nose by myself, and an additional strain, U, derived from a culture, which was very kindly furnished by Dr M. Gordon: it was obtained by him from the Hertfordshire epidemic and agreed in all particulars with my own strains.

5. *Catarrhalis-like organisms.* Of these I have isolated three strains from the nose:

Strain Y, from a catarrhal nose, which also yielded typical catarrhalis: this strain was very much like the type, but was constantly small and of unusually uniform size.

Strain Q, isolated from a normal nose, in company with the pneumococcus: it was a small coccus and its sugar reactions differed from catarrhalis.

Strain V, obtained in pure culture from the nose of a boy, where it was found in a purulent discharge which had been present since he had scarlet fever some months before. It resembled *M. catarrhalis* except in its sugar reactions.

I have grouped together the 19 strains which I consider to be *M. catarrhalis,* although they present minor differences as to vigour of growth and power of surviving on agar.

TABLE III.

	Gelatin at 20° C.	Gram.	Broth	Ascitic broth	Emulsion	Spon. agglutination	Agglutination with meningo-serum	Maltose broth	Glucose broth	Galactose broth	Saccharose broth	Laevulose broth	Lactose broth
Meningococcus XII	0	0	Turbid	Turbid	Good	0	+	A	A	A	–	A	–
,, XVII	0	0	,,	,,	,,	0	+	A	A	A	–	A	–
,, XVIII	0[1]	0	,,	,,	,,	0	+	A	A	A	–	A	–
,, XIX	0	0	,,	,,	,,	0	+	A	A	A	–	A	–
,, XXIX	0	0	,,	,,	,,	0	+	A	–	–	–	A	–
,, M 38	0	0	,,	...	...	...	...	A	A	A	–	A	–
4M	+	0	,,	...	Good	0	–	–	–	–	–	–	–
Catarrhalis 19 strains	+	0	Clear & granules	Turbid & ring	Bad	+	...	–	–	–	–	–	–
,, strain U	+												
Catarrhalis-like													
,, Q	Fair	0	Clear sand	...	Bad	...	...	A	A	A	A	A	–
,, V	Good	0	Clear granules	...	,,	+	...	A	A	A	A	A	A
,, Y	Good	0	Clear granules	...	,,	+	...	–	–	–	–	–	–
Gonococcus[2]	0	0	...	Clear & granules	Fair	+	...	–	A	A	–	–	–

A = Acid production.

[1] XVIII did on one occasion grow on gelatin.

[2] Ascitic fluid was added to the sugar-broth for growing the gonococcus.

As regards morphology, *M. catarrhalis* is more uniform in appearance and better stained by methylene blue than meningococcus.

The growth of *M. catarrhalis* on agar at 37° C. on first isolation was rather feeble, but after a few sub-cultures it became more vigorous; the colonies on agar were firm, so that the platinum wire in making a sub-culture broke solid pieces off or detached the whole colony, whereas the meningococcus colonies were of a slimy or honey-like consistency. On gelatin at 20° C. the growth, though often feeble at first, later became whitish and more vigorous. The gelatin was not liquefied; if the tube was incubated at 37° C. the appearance of the culture resembled that in broth, and on cooling the medium completely solidified. In ordinary *broth* cultures the medium always remained clear (unless shaken) with a coarsely granular or sand-like deposit at the bottom, usually suspended in a mucus-like ball. These cultures in broth resembled those of the gonococcus in ascitic-broth, but when ascitic fluid or sugar was added to broth and *M. catarrhalis* inoculated, growth always caused turbidity.

The catarrhalis-like organisms Y, Q, and V, from the nose, all grew in broth as a granular deposit and left the medium clear: Q forming fine sand which adhered to the glass: V forming coarse granules in a glairy ball. 4M caused uniform turbidity like meningococcus.

Carbohydrate peptone broths made with 75 % peptone water, 25 % peptone beef broth, and 1 % of carbohydrate were used.

M. catarrhalis and the strains Y and 4M formed no acid in glucose, maltose, laevulose, galactose, cane sugar or lactose. Strain V formed acid in 24 hours in all these sugars, and Q in 24 hours in all of these except lactose.

Good uniform emulsions of *M. catarrhalis* and the catarrhalis-like organisms were very difficult to obtain, but the meningococcus and strain 4M were easily emulsified.

Agglutination with a specific serum as a means of differentiating *M. catarrhalis* is of little service, since all the strains cultivated by me agglutinated spontaneously, but the serum of a rabbit repeatedly inoculated intravenously with cultures of *M. catarrhalis* did not agglutinate the meningococcus or strain 4M. The gonococcus and strains Y and V all agglutinated spontaneously.

The character of spontaneous agglutination appears to be so constant as to form an important point for differentiating these groups of Gram-negative cocci, especially as the strains of meningococcus

examined by me never agglutinated spontaneously, nor can I find that other workers have observed this occurrence.

Conclusions.

1. Gram-negative cocci derived from the nose can be divided into several different races, which require very careful culture for their identification.

2. *M. catarrhalis* is present very frequently in the normal nose, especially in the young and more especially in infants.

3. Its frequency does not appear to be greater in ordinary catarrhal states than in non-catarrhal. In this respect it differs from the pneumococcus and Hoffman's bacillus.

My thanks are due to Dr A. Macfadyen for having suggested the work contained in this paper and for his help and advice, and to Dr A. E. Boycott for much help and advice throughout.

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The Quantitative Estimation of Small Quantities of Nickel in Organic Substances.

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(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—Read February 1, 1906.)

(From the Chemical Department of the Lister Institute of Preventive Medicine.)

In the course of an investigation into the toxic action of certain nickel compounds, it was found necessary to devise a method of detecting and estimating nickel, when included in animal tissue, in quantities not exceeding a few milligrammes per cent.

A method has therefore been worked out, which, although in many respects only differing from the usual methods in virtue of slight alterations of detail, is capable of demonstrating extremely small quantities of nickel accurately. The method may be divided into three stages: (1) The Ashing; (2) The Separating; and (3) The Estimating stages.

1. *Ashing.*—The substance to be examined must be placed in a porcelain crucible (platinum is unsuitable, as a considerable loss of nickel takes place, probably by an alloy of platinum and nickel being formed) and evaporated to dryness over a water bath. If the substance be solid, it should be cut up into small pieces. The crucible is then heated carefully with a Bunsen flame, but it may be wise to further dry in a hot air oven or on a sand bath before this. Then it is burned over a Fletcher burner, and lastly fully incinerated in the blow-pipe flame. With some care, it is possible, as a rule, to oxidise fully all the carbon, without recourse to any foreign material. The crucible is then placed on the water bath, and 10 c.c. of pure hydrochloric acid are added and allowed to evaporate to dryness, this process being repeated. The residue is then extracted with water to which a small quantity of hydro-

chloric acid is added. For this purpose, 2 c.c. of a four times normal acid are generally employed. The extract is then filtered. The ash so obtained is practically completely soluble.

Ashing by Kjeldahl's method, or better, with sulphuric and nitric acids, can also be employed, but has two disadvantages over the simple incineration method: Firstly, it takes longer; and secondly, it introduces foreign salts, which should, if possible, be avoided.

2. *Separation.*—Firstly, it is necessary to get rid of the iron and at the same time of the phosphates. Those tissues, which contain iron in excess, *e.g.*, blood, may be treated by precipitation with excess of ammonia and filtration. The process should be repeated three times, the precipitate being redissolved each time with the same quantity of acid as was used for the extraction. If some of the iron separates out from the filtrate on being evaporated, it may be necessary to refilter before dryness is reached. When the substance contains little or no iron, it is necessary to add a sufficiency to combine with the quantity of phosphates present. If the phosphates are present in excess, the following method is employed. The cold solution is made neutral to litmus or very faintly acid with ammonia. An excess of ammonium acetate is then added (as a rule 8 to 10 c.c. of a 10-per-cent. solution suffices), and sufficient ferric chloride to colour the supernatant fluid yellowish-red. The mixture is then boiled, when all the iron separates out as phosphate and basic acetate. For those tissues yielding large quantities of phosphates, *e.g.*, liver, the amount of ferric chloride necessary is comparatively large, and some difficulty may be experienced with the filtration. The only possible help is obtained by using two or more filters. The washing of the precipitate must be carried out carefully, in spite of the considerable loss of time. Precipitation by ammonia may be carried out for the three subsequent repetitions. The precipitate is each time dissolved in the smallest possible quantity of acid.

After the united filtrates have been evaporated to dryness, the residue is again dissolved in water, and dilute hydrochloric acid added, about 6 c.c. of four times normal acid being usually sufficient. Sulphuretted hydrogen is then passed through the hot solution for at least half an hour, and it is then allowed to stand for a time, as the sulphides, which form in acid solution do not always readily separate out. The liquid is then filtered and the precipitate well washed with sulphuretted hydrogen water. The filtrate is again evaporated to dryness, re-dissolved in a little water on the water bath and then a solution of pure sodium hydrate is added in successive portions to the hot liquid, until no more ammonia comes off. Care should be taken to use as little sodium hydrate solution as possible, as every sample in the market

contains small traces of iron. The nickel is thus precipitated in the form of the hydrate, and this is converted into nickel sesquioxide by the addition of 1 or 2 c.c. of bromine to the cold mixture. The nickel oxide is then collected by filtration, and after having been well washed, is dissolved in hydrochloric acid, and the solution evaporated to dryness to remove the excess of acid, and the residue re-dissolved in water with a faint trace of acid, in order to prevent the formation of basic salts. The solution is finally made up to a definite volume.

In the process of separation, no especial difficulties save the management of the voluminous iron precipitate, are met with as a rule. At times an insoluble residue is found on the filter paper when the oxide is dissolved. This is a trace of a sulphide of copper or another metal of this group, which has escaped precipitation by sulphuretted hydrogen in acid solution.

3. *Estimation.*—The usual method of quantitative estimation of nickel colorimetrically is carried out with ammonium sulphide, but it has been found that sharper results can be obtained by employing α-dimethylglyoxime $CH_3.C(N.OH).C(N.OH).CH_3$, which was recently shown by Tschugaeff to form a scarlet red compound with nickel in the presence of ammonia.*

For this purpose, a saturated solution of the reagent in absolute alcohol is prepared, this is diluted with water until a little of the compound separates out, and alcohol is then added until complete solution takes place. The fluid to be tested and a standard solution of nickel sulphate are placed in burettes. A measured quantity of the fluid is then run into a Nessler tube and to this 0·5 c.c. of a 10-per-cent. solution of ammonia and the same quantity of the dimethylglyoxime solution are added and the whole made up to 30 c.c. It is better first to add the ammonia to the nickel solution, then the dimethylglyoxime, and then allow the colour to develop before diluting up to the 30 c.c. mark. All the solutions must be cold. The fluid becomes coloured pinkish red, the depth of the coloration depending on the quantity of nickel present. The most convenient quantity to work with is about 0·08 to 0·01 milligramme. The colour is then compared with that produced by varying quantities of nickel from the standard solution. The determination is not complete until a quantity has been found, which gives a colour which is just too pink, and a second quantity the colour of which is just appreciably less pink, than the fluid to be tested. The quantity of nickel contained is then calculated as the amount midway between the two tubes. With a little practice, it is quite easy quickly to determine very small differences of colour. The estimation should be concluded as rapidly as is compatible with accuracy,

* 'Deut. Chem. Ges. Ber.,' 1905, vol. 38, p. 2520.

as, after a short time, the nickel compound with dimethylglyoxime separates out of the coloured solution as a precipitate.

The advantages of this method over the ammonium sulphide method are: (1) small traces of iron do not interfere with the final colour, nor with the sharpness of the method; (2) smaller quantities of nickel can be accurately estimated; and (3) it is easier to work in a bad light with the pink than with the brown colorimetric determination.

Dealing with the colorimetric test alone, with solutions of pure nickel sulphate, the smallest quantity which gives the reaction is 1/1000 milligramme. To detect such a small amount, the solution must be placed in the Nessler tube, then the ammonia and the solution of dimethylglyoxime added, when one can recognise the characteristic pink colour, and, lastly, the fluid is made up to 30 c.c.; on comparing this with distilled water, a faint but distinct difference is seen. 3/1000 milligramme gives a recognisable pink colour in 30 c.c. of fluid. Working with 0·07 milligramme, differences of 1/1000 milligramme can be recognised with a little practice. This represents a potential error of + or —0·7 per cent.

In test analyses, serum or blood with nickel sulphate added, the experimental error was kept as low as 2 per cent., using about 1 milligramme of nickel. For example, about 30 grammes of blood were placed in a crucible and 0·9 milligramme of nickel, in the form of the dissolved sulphate, was added. After ashing, extracting, removing the copper and iron groups, and precipitating the nickel in the form of the sesquioxide, etc., the final fluid was made up to 30 c.c.; 2 c.c. of this fluid were compared with varying quantities of a solution containing 0·01 milligramme of nickel per cubic centimetre. It was found that 5·9 c.c. gave a colour, which was just too pink, and 5·8 c.c., a colour, which was not pink enough, so that the 2 c.c. contained 0·0585 milligramme of nickel, and the whole solution 0·88 milligramme. This represents a loss of 0·02 in 0·9, or about 2 per cent.

Electrolysis is only to be preferred when large quantities of nickel are to be measured, while the method described above is intended for the recognition and measuring of quantities of nickel not exceeding a few milligrammes.

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THE EFFECT OF ARTERIAL OR VENOUS OBSTRUCTION UPON THE NUTRITION OF THE LIVER CELLS

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(*Received November 13th,* 1906)

Introduction

These experiments were undertaken in order to ascertain more definitely the relative parts played by the arterial and venous blood, which supply the liver, in the nutrition of the liver cells ; and attention was directed more especially to the appearance of fatty changes in these cells. Widely differing views have been held by various writers as to the extent to which the hepatic artery directly supplies arterial blood to the liver cells. The observations of Cohnheim and Litten led them to believe that no arterial blood entered directly the capillary network of the lobules ; they supposed that the hepatic artery supplied blood to the connective tissue framework of the liver, and nourished the walls of the portal vessels running in that framework. Rindfleisch, however, concluded—on the ground of the changes occurring in amyloid disease—that the hepatic arterioles joined the capillary network in the middle zone of the lobule, and that the outer zone received only portal blood.

Recently, Kowalewsky and Opie have independently come to the conclusion that both the artery and the portal vein supply the entire lobule.

Cohnheim and Litten, working with rabbits, found that ligature of the hepatic artery caused necrosis of the entire liver, and death in twenty-four hours. After tying the artery going to a single lobe, the animals lived two or three days. The lobe was necrotic, and showed scattered haemorrhages ; no fatty change was observed.

stained with Soudan III; other pieces were treated by Marchi's method, or stained with the ordinary dyes.

B. Chemical.—The pieces of liver taken for the fat determinations were freed from blood, dried, and powdered, and the powder dried in vacuo at 100° C. The dry powder was weighed and extracted with chloroform in a Soxhlet apparatus after being boiled for a short time in alcohol, according to the method recommended by Rosenfeld. The extract so obtained was then boiled with alcoholic potash, and the fatty acids insoluble in water, set free by acidification of a dilute aqueous solution of the soaps, were collected, taken up in light petroleum, freed from solvent, and weighed. The weight of the insoluble higher fatty acids thus obtained was calculated in percentage of the total solids of the tissue.

Results

A. Ligature of the Hepatic Artery

Owing to the presence of small collateral arteries supplying the liver in cats it is difficult, perhaps impossible, absolutely to shut off all arterial blood from the liver. Consequently, the morbid changes observed vary in degree corresponding to the extent to which arterial blood is excluded. Ligature both of the main artery and of all visible collaterals causes death in twenty-four hours, and very intense changes in the liver cells; ligature simply of the main artery modifies the nutrition of the liver cells to a variable extent in different animals. The pathological alterations vary greatly in degree, and in any case last only for a few weeks, since recovery takes place by the development of the collateral circulation.

The morbid appearances to be described are the result, therefore, of a partial (and variable) deprivation of the arterial blood supply.

I. Histological Changes.—The two points observed which seem of most importance are in the first place the increase in the amount of fat visible in stained sections, and secondly the absence of gross necrosis of the liver. When death occurred within twenty-four hours the liver looked abnormally pale to the naked eye; no haemorrhages or necrotic areas were visible.

Microscopically the portal vessels supplying the lobules were very full of blood; some, but not all, of them were thrombosed. The columnar arrangement of the cells had to some extent disappeared. The cell-nuclei stained well throughout the lobule, except in the inner zone, where some necrotic cells were visible, which stained very badly. There was, however, no necrosis of the lobule as a whole, although all the cells were obviously severely damaged. In the outer zone of the lobules the protoplasm was shrunken, granular, and apparently free from glycogen; in the inner zone many of the cells contained large vacuoles; sections stained with Soudan III showed abundance of fat in the inner zone, corresponding to the vacuoles observed in paraffin sections.

In one experiment, in which the occlusion of arterial blood was almost complete, there was no increase of fat histologically; even in this case there was no general necrosis.

If the animals survived more than twenty-four hours they were killed by chloroform from two to twelve days after the operation. In such experiments the liver looked pale and fatty on inspection.

Microscopically, the outer and middle zones of the lobules were normal in appearance; the inner zone contained abundance of fat. A few days after the operation the fat existed chiefly in the form of large globules; later on it was found as small drops only, and in gradually diminishing amount. In animals killed two or three days after the operation scattered necrotic cells were visible in the inner zone close to the intralobular vein; some of the cells also contained granules of brown pigment, probably bile.

With one exception, the capillary network was not unusually engorged with blood, and the general appearance of the liver was not that of infarction. In one experiment the animal was killed twenty-six days after the operation. The liver appeared normal both microscopically and on section. This may have been due to recovery by the establishment of a collateral circulation, or the animal may, in the first instance, have possessed a good collateral blood supply, so that ligature of the main hepatic artery only slightly damaged the liver.

It should be pointed out that in normal cats the amount of fat found (histologically) in the liver is extremely variable; in some livers the entire lobule may be full of drops of fat, in others the fat may be almost confined to the outer or inner zone, and in others hardly any fat may be visible. It is most desirable, therefore, to snip off a small piece of liver as a control at the time of the operation; even this, however, is not satisfactory if the control piece is full of fat.

Metabolic changes have been described in animals after ligature of the hepatic artery. In our experiments the urine contained traces of sugar for a day or two after the operation; later the urine was normal, and the relation of urea to the total nitrogen was unaltered. For a few days the animals lost weight; subsequently, however, they regained or even exceeded their original weight.

B. Ligature of the Portal Vein

The lobe which was deprived of its portal blood never showed gross necrosis, nor did any appreciable increase in the amount of fat take place.

The animals usually survived, but one or two died within 24 hours. In such cases the columnar arrangement of the cells was lost; the cells throughout the lobule stained badly, and many of them were necrosed, and the lobules as a whole looked atrophied, as though many cells had broken down altogether and disappeared. These necrotic cells were scattered throughout the lobule, and were not confined to any one zone. A few globules of fat were visible, but they were not numerous.

Four days after the ligature the outer zone appeared normal; in the middle and inner zones the nuclei stained well, but the cell substance stained very lightly indeed. Sections stained with Soudan III showed practically no increase of fat. Eighteen days after the ligature the liver was tough and firm, the portal canals were very obvious, and the lobe appeared atrophied. Microscopically, the liver showed a general fibrosis round the arteries and ducts of the portal canals, and the interlobular connective tissue was everywhere increased in amount.

The inner zone was represented chiefly by extravasated blood; the liver cells were scanty, stained badly, and often contained pigment granules. There was no increase in the amount of fat.

The analytical determination of the amount of fat was carried out by the method described above in altogether four animals in which the hepatic artery was tied, and in one in which the vessel ligatured was the branch of the portal vein supplying the right lobe.

In one only of these experiments was the absolute amount of fat in the liver found to be increased after the ligature.

Experiment No. 4 [January 24].—The branch of the hepatic artery to the right lobe was tied, and two days later the fat estimated in this lobe and in two portions of the rest of the liver. In the former the insoluble higher fatty acids amounted to 21·3 per cent. of the dry substance, in the latter to 14·8 and 14·5 per cent. respectively.

But since the characteristic microscopic appearance of fatty change was not evident in this case, the increase in the fat here does not help us to determine the nature of this change.

In all the other cases the amount of fat was either unaltered or diminished, and it was diminished in that case in which the histological appearance of increase was most marked.

Experiment No. 1 [October 28].—The left lobe, removed at the time of the operation in which the hepatic artery and its branches were tied, contained 24·6 per cent. of fat; two days later two samples of the liver contained 19·15 and 19·5 per cent. respectively.

Experiment No. 3 [December 8].—The branch of the hepatic artery supplying the left lobe was tied; two days later this lobe contained 10·5 per cent., and the rest of the liver 10·4 and 10·25 per cent. in two portions taken for analysis.

Experiment No. 5 [July 3].—The left lobe, removed when the hepatic artery and all its branches were ligatured, contained higher fatty acids, amounting to 11·2 per cent. of the dried liver substance. The liver at death on the next day contained 9·7 per cent.

Experiment No. 2 [December 4].—The branch of the portal vein to one lobe was tied. Two days later this lobe contained 13·7 per cent., and the portions of the rest of the liver analysed 13·0 and 13·6 per cent. respectively.

Conclusions

(1) Ligature of a branch of the portal vein is followed by atrophy of the liver cells, and by fibrosis of the interlobular connective tissue, probably as a replacement fibrosis. It is possible, therefore, that in some cases of hepatic cirrhosis associated with portal thrombosis the thrombosis precedes rather than follows the cirrhosis.

(2) Ligature of the hepatic artery produces (*a*) necrosis of scattered cells in the inner zone, but no general necrosis of the liver, even when the animals die within twenty-four hours, and (*b*) considerable injury to the whole of the inner zone, as indicated by the increase of fat (histologically) in the cells.

As regards the necrosis, it is probable that complete occlusion of all arterial blood would cause general necrosis of the liver ; in our experiments this condition was never absolutely fulfilled, and the bulk of the liver cells, though severely damaged, were not killed. There is no doubt that a partial occlusion of arterial blood, if associated with septic infection, can set up necrosis of the liver ; and the combination of these two factors may account for the results obtained by some observers, as was pointed out by Dujarier and Castaigne.

The possible sources of the apparent excess of fat are (1) an infiltration from without ; (2) the formation of fat from proteid by the process of ' fatty degeneration ' hypothesised by Virchow ; and (3) the setting free in a form recognisable by the microscope of fat previously bound up in some complex combination in which it is not detectable by microscopic examination—a process comparable with the liberation of simple fats from myelin in nerve degeneration.

The chemical analyses show clearly that there is no *real* increase of fat in the liver ; the fat, therefore, cannot be either an infiltration or a product of ' fatty degeneration ' of proteid. We consider that when the liver cells are deprived of arterial blood a process of autolysis occurs whereby pre-existent fat recognisable by chemical analysis is set free in a form which stains with Soudan III. It seems clear that this autolysis results from the lack of oxygen, since it is not produced by portal obstruction ; and as the deficiency of oxygen is most marked in the centre of the lobule, the inner zone is chiefly affected.

A very similar condition of the liver (histologically) has been observed by Drummond in animals, and by Langdon Brown in man after intravenous injections of adrenalin ; it may be surmised that in this case, too, the effect is partly the result of a deficient arterial blood supply to the liver.

There was nothing to suggest that the fatty changes were due to toxic or infective causes, since the animals rapidly recovered after the operation, and showed no signs of sepsis.

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THE STAINING ACT: AN INVESTIGATION INTO THE NATURE OF METHYLENBLUE-EOSIN STAINING

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(*Received July 28th*, 1906)

The various theories of the nature of the act of staining of animal cells, tissues and products, which have been put forward by different writers fall into three groups: (1) that the process of staining is a chemical reaction between the dye on the one hand, and the cell or tissue elements on the other; (2) that in staining the dye is adsorbed by the tissue elements stained; and (3) that the dye is present in the form of a solid solution in the stained material. The problem remains, however, still unsettled, further data respecting individual stains being much to be desired.[1]

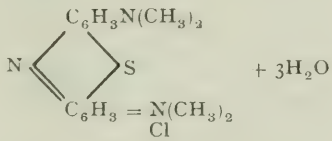
For studying the nature of an individual staining act methylen-blue-eosin was selected as being a dye which is peculiarly suitable for the purpose. This substance is a salt which is formed by the union of the basic radical of methylenblue and the acid radical of eosin. When it is allowed to act upon cells or tissues some of the elements stain blue, others red. For example, with an alcoholic or aqueous solution of methylenblue-eosin a polynuclear leucocyte exhibits blue staining of the chromoplasm of the nucleus, while at the same time the oxyphile granules of the cytoplasm stain deep red, precisely similar blue staining being obtained with methylenblue and the red staining with eosin. In the case of methylenblue-eosin, provided that hydrolysis of the dye can be excluded, the act of staining can only be explained as due to a chemical reaction between dye and cell elements. If hydrolysis is not avoided then both adsorption and solid solution of the free acid and the free base may possibly occur and produce the staining effect.

1. Cf. W. M. Bayliss, 'On some aspects of adsorption phenomena,' *The Bio-Chemical Journ.*, 1906, Vol. I, p. 175.

As it seemed not improbable that an investigation of the colour and spectroscopic characters of methylenblue-eosin might throw light upon the presence or absence of hydrolysis of the dye, this was first undertaken. In this investigation the condition of methylenblue-eosin in solution in alcohol forms the primary object of study, but attention is also given to various points respecting alcoholic and watery solutions of methylenblue, eosin and allied substances, since these data have a direct or indirect bearing on the subject of enquiry. It will be convenient to commence with the latter two dyes.

Colour and Spectroscopic Characters

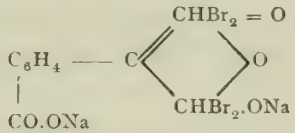
Methylenblue is tetramethyldiamidothiodiphenylamin chloride, having the formula



and the molecular weight 371, or after freeing from water at 100° C., 317·36. This salt crystallises in dark blue microscopic plates; from solution in water it is precipitated by sodium chloride and by zinc chloride. The form employed in the present work was the double salt with zinc chloride, having the formula $2C_{16}H_{18}N_3SCl + ZnCl_2 + H_2O$, and the molecular weight 787·96, or when anhydrous 770·08. The sample employed (rectif., for injection *intra vitam*, Grübler) contained 9·12% S, (theory requiring 8·17%). Methylenblue, if allowed to separate out slowly from alcoholic or aqueous solution crystallises in needles and prisms having a bronze lustre on the surface, but when rapidly evaporated it forms a film, which under the microscope does not exhibit crystals, but is amorphous in character. In alcoholic and in aqueous solution of ·0001 N to ·00001 N concentration (·0079% to ·00079%) it forms a characteristic blue liquid, no difference of colour between alcoholic and aqueous solutions being recognisable to the naked eye, though on spectroscopic examination a marked difference is recognisable. Thus in alcoholic solution of $2·80 \times 10^{-5}$ N

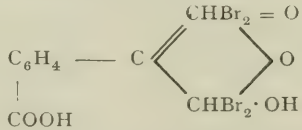
concentration, in a layer 1 cm. thick, a very dark band appears in the red, while between this band and the solar line D a lesser degree of absorption occurs (Fig. 1, A) ; in aqueous solution a narrower very dark band is seen and a second less dark band running up to D (Fig. 1, H).

Water soluble eosin, the sodium salt of tetrabromfluoresceïn, $C_{20}H_6Br_4O_5Na_2 + 5H_2O$, to which the constitutional formula



has been assigned,[1] has a molecular weight of 776·2 or, without water of crystallisation 686·2. The sample employed contained after drying 41·7% Br, theory requiring 47·2% Br. From watery solution it crystallises out in triclinic red crystals, exhibiting a greenish surface lustre. Its colour in ·001 N concentration (·078%) in alcohol is yellowish red with a strong light-yellowish green fluorescence ; in water it is of a darker brownish red colour and shows a slighter olive green fluorescence. In ·00001 N concentration the same difference in respect of fluorescence is seen, but the colour in alcohol is a vivid pink, in water a slightly brownish pink.[2] The spectrum in alcohol in $2{\cdot}45 \times 10^{-5}$ N concentration, in a layer 1 cm. thick shows a very dark band in the green about the solar line E, together with a second lighter band towards F, as shown in Fig. 1, B ; in aqueous solution of the same concentration a broader very dark band nearer the blue end of the spectrum is seen, but no second absorption band is observed (Fig. 1, F).

Alcohol soluble eosin ($C_{20}H_8Br_4O_5$), the free acid corresponding to the foregoing salt, receiving the formula



1. The exact constitution of the eosin molecule is not yet established. In accordance with current views of the nature of the chromophore group, the quinone ring is exhibited in the formulae given.

2. The above statements refer to the colour of solutions in test-tubes. A layer of eosin solution of ·01 N concentration, ·5 mm. thick, has the same colour as a layer of eosin solution of ·0002 N, 25 mm. thick.

has a molecular weight of 643·0. The sample used contained after drying 47·6% Br. theory requiring 48·3% Br. From alcoholic solution it crystallises on slow evaporation in yellowish red crystals,

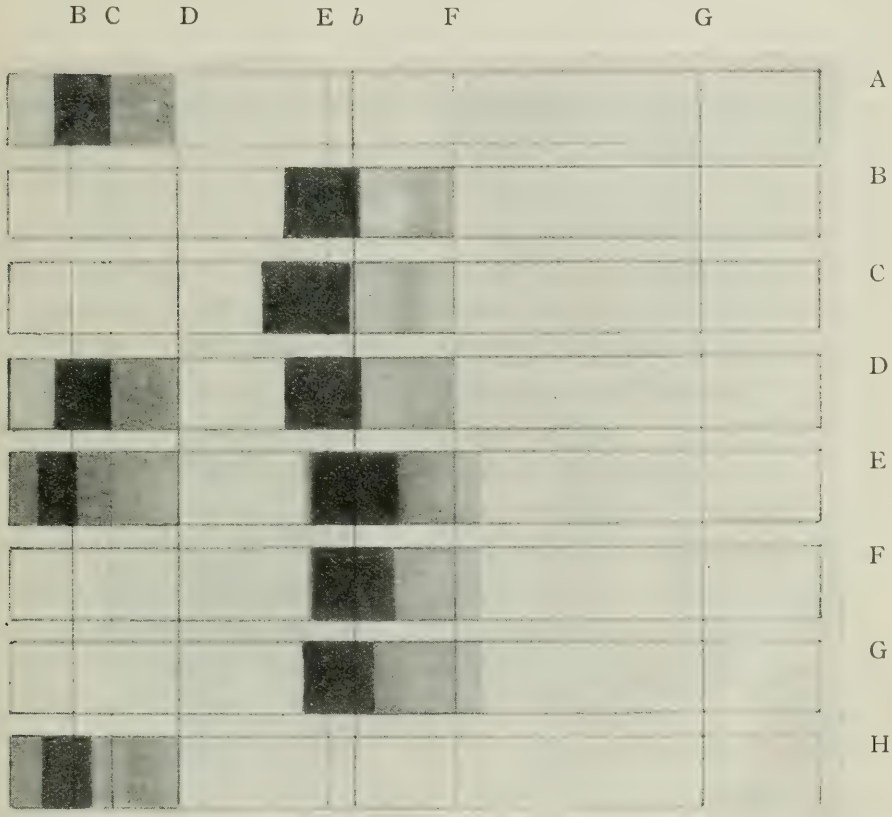


Fig. 1. Absorption spectra. In absolute alcohol: A, methylenblue, in $2{\cdot}78 \times 10^{-5}$ N concentration; B, water soluble eosin, in $2{\cdot}45 \times 10^{-5}$ N concentration; C, alcohol soluble eosin, in $2{\cdot}50 \times 10^{-5}$ N concentration; D, methylenblue-eosin, in $2{\cdot}80 \times 10^{-5}$ N concentration. In water: E, methylenblue-eosin, in $2{\cdot}80 \times 10^{-5}$ N concentration; F, water soluble eosin, in $2{\cdot}45 \times 10^{-5}$ N concentration; G, alcohol soluble eosin, in $2{\cdot}50 \times 10^{-5}$ N concentration; H, methylenblue, in $2{\cdot}78 \times 10^{-5}$ N concentration. The liquids were viewed in a layer 1 cm. thick.

having the formula $C_{20}H_8Br_4O_5C_2H_6O$; on rapid evaporation a film forms in which no crystals are recognisable; from alcoholic solution to which hydrochloric acid has been added, in red alcohol-free

crystals. The colour of a ·001 N solution in alcohol is a deeper pink than that of the sodium salt in the same solvent, and is accompanied by a slight yellowish green fluorescence; in ·0003 N concentration in water its colour is a deeper brownish pink with a slight olive-green fluorescence. In alcohol in ·00001 N concentration it shows a brilliant slightly violet pink colour, and in water in the same concentration a slight brownish tint is added, the same difference as before in respect of fluorescence being seen. The spectrum of alcohol soluble eosin of $2{\cdot}50 \times 10^{-5}$ N concentration in a layer 1 cm. thick in alcohol (Fig. 1, C) shows a very dark broad band in the green and to the side of this, nearest the blue end of the spectrum, a second lighter narrower band, both bands being slightly nearer the red end of the spectrum than the corresponding bands of water soluble eosin, whose dark band is not so wide. In aqueous solution in the same concentration (Fig. 1, C), the absorption spectrum differs from that of the sodium salt in water in the same concentration (Fig. 1, F), in the diminished width of the dark band which is displaced towards the red, an exceedingly faint second absorption band being seen towards F.

Methylenblue-eosin was first prepared by Romanowsky[1] (1891), who observed that when concentrated solutions of methylenblue and eosin were mixed a precipitate formed, which, when dissolved, furnished a selective stain for the nuclear chromoplasm of the Plasmodium malariae. Ziemann[2] (1898), who made use of the same stain, observed that the precipitate was soluble in excess of the methylenblue, as also of the eosin solution. Rosin[3] (1899), found that similar precipitates were obtainable from mixtures of other so-called basic and acid dyes. None of these observers, however, investigated the chemical composition of Methylenblue-eosin. Jenner[4] (1899), who made use of this dye for staining blood-films, found that it dissolved to the extent of about ·5% in the methyl alcohol, was less soluble in ethyl alcohol, and

1. *Zur Frage der Parasitologie und der Therapie der Malaria,* 1891.

2. 'Über Malaria und andere Blutparasiten,' etc., Jena, 1898, Fischer. Also *Centralbl. f. Bakteriol.*, 1898, No. 25.

3. 'Über eine Gruppe von Anilinfarbstoffen, ihre Bedentung für die Biochemie der Zelle und ihre Verwendbarkeit für die Gewebsfärbung,' *Berl. kl. Wochenschr.*, 1899, 36 Jahrg., p. 251.

4. 'A new method for rapidly fixing and staining blood,' *Lancet,* 1899, Vol. I, p. 370.

much less soluble in water[1]. He regarded methylenblue-eosin as a salt, since he observed that, no matter what the proportions in which methylenblue and eosin were mixed might be, the precipitate formed always possessed the same melting point, namely, 227° C. The precipitate, he further observed, consisted of brilliant grass-green crystals if alcoholic solutions were used (with rapid evaporation a film forms in which no crystals are recognisable), but was amorphous and showed a metallic green colour if aqueous solutions were employed. Jenner did not determine the composition of methylenblue-eosin, and though he mentions that the most abundant precipitate is formed when methylenblue and eosin are mixed in the proportion of 8·0 to 8·3 parts of the former and 10 parts of the latter, yet this statement, in the absence of further details as to the composition and purity of the methylenblue and eosin employed, does not enable a conclusion to be reached as to the composition of the precipitate. The absence of a sharp indication of accurate admixture of methylenblue and eosin renders this method of attempting to determine the relative proportions required unsatisfactory. It may be pointed out that if the proportion of two molecules of methylenblue ($2C_{16}H_{18}N_3SCl + ZnCl_2 + H_2O$, mol. wt. 788) and one of eosin ($C_{20}H_6Na_2Br_4O_5 + 5H_2O$, mol. wt. 776) are employed, the ratio of the former to the latter, by weight, would be 10·2 : 10 ; if the proportion is one and a half to one, the ratio becomes 7·55 : 10.

The composition of methylenblue-eosin[2] was therefore further investigated, two methods being employed. In the first place by means of the colorimetric observations described below it was ascertained that the proportions in which methylenblue and eosin react to produce methylenblue-eosin were two molecules of the former ($C_{16}H_{18}N_3SCl$) and one molecule of the latter ($C_{20}H_6Na_2Br_4O_5$). In the second place, by ultimate analysis, the percentages of sulphur and bromine respectively in methylenblue-eosin dried to constant weight at 100° C. were found to be 5·12 and 23·50, a second analysis giving

1. It dissolves in water to the extent of about ·02 per cent. at 16° C.

2. Methylenblue-eosin is quite free from chlorine and zinc on the one hand, and from sodium, potassium and ammonium on the other, so that the possibility of its being an admixture of methylenblue and eosin is altogether excluded.

5·35 and 23·84 The formula $(C_{16}H_{18}N_3S)_2C_{20}H_6Br_4O_5$ (mol. wt. 1204·4), which requires 5·28% of sulphur and 26·34% of bromine, therefore represents the required relation.

The colour of methylenblue-eosin in absolute alcohol in ·001 N concentration (·12%) is dark purple with a strong dark yellowish green fluorescence; in ·00001 N concentration, bluish purple with the same strong fluorescence. In aqueous solution in the latter concentration its colour is reddish purple with a very slight dark olive green fluorescence. In alcoholic solution of $2·8 \times 10^{-5}$ N concentration in a layer 1 cm. thick, methylenblue-eosin exhibits a spectrum (Fig. 1, D) showing absorption bands both in the red and the green, the former being identical with that of methylenblue in corresponding concentration (Fig. 1, A), the latter with that of water soluble eosin in corresponding concentration. In aqueous solution in the above concentration and thickness, a different absorption spectrum is seen, in the red a very dark band touching Fraunhofer's line B being seen with a lighter absorption area on each side; while in the green a very dark band centred about *b* is seen, with a lesser degree of absorption towards F but without a second lighter band as in alcohol. Methylenblue-eosin, therefore, in water shows bands which are different from those exhibited by methylenblue and eosin respectively in aqueous solution.

If methylenblue-eosin is dissolved in a test-tube of alcohol in amount sufficiently small to produce a convenient depth of colour[1], no difficulty is experienced in ascertaining the respective amounts of methylenblue and of water soluble eosin, which require to be added to a second tube of alcohol in order to produce the same quality and intensity of colour, which the first exhibits. If the eosin is employed in the alcohol soluble form, the colour of the first tube can be very nearly matched but complete equality of colour is not attainable. In water, also, a methylenblue-eosin solution may similarly be matched with methylenblue and water soluble eosin, but when alcohol soluble eosin is employed matching is attended with difficulty, for although

1. Only dilute solutions can be employed owing to the intense colour of the dyes. The concentrations given in Table II will be found suitable in most cases.

immediately after mixing the latter two dyes in suitable proportions a fair equality of colour is obtainable yet the colour rapidly changes, the mixed liquid becoming bluer until at the end of twenty-four hours it has assumed the appearance of a methylenblue solution, the eosin having separated out in the form of a red precipitate, which has settled at the bottom of the test-tube. It appears, therefore, that in this case methylenblue-eosin is not formed, a simple mixture of the two dyes resulting and the alcohol soluble eosin being after a time 'salted out' by the methylenblue. In two cases, however, namely when methylenblue and water soluble eosin are employed in alcohol or in water, equality of colour is attainable both as regards tint and depth. The smallest variation of colour which, under the most favourable conditions in respect of light, of background and of freedom of the eyes from fatigue, is recognisable with certainty is represented by a variation of about 2½% in the amount of the solutions of methylenblue or water soluble eosin required to produce equality. When equality of colour has been attained the spectra are found to be identical also. The concentration of methylenblue and of water soluble eosin, which was found after repeated trials to give equality, is shown in Table 2, Exps. 1 and 2. The composition of the methylenblue-eosin employed has been already referred to (p. 411); the sample of methylenblue, $2(C_{16}H_{14}N_3SCl)\cdot ZnCl_2$, used was found on analysis (p. 407) to contain 9·12% of sulphur (theoretically 8·17% is required), and the eosin salt, $C_{20}H_6Br_4O_5Na_2$, contained (p. 408) 41·7% of bromine (theoretically 47·20%). It is clear, from the results in Table 2, that in the formation of methylenblue-eosin two molecules of zinc-free methylenblue, $C_{16}H_{14}N_3SCl$, and one molecule of eosin take part. It is to be noted that the slight defect of the eosin radical already noted in the methylenblue-eosin employed has its counterpart in the concentrations required to match ($\cdot 48 \times 10^{-5}$ N instead of $\cdot 54 \times 10^{-5}$ N, Table 2, Exps. 1, 3, 5, 7). The cause of these differences of concentration obtaining when equality of colour was reached is presumably to be attributed to the formation of small amounts of leuco or other derivatives of feeble colour intensity; no indication of the presence of such compounds is, however, afforded by the analyses made (pp. 407-

411). It would appear that methylenblue and methylenblue-eosin are more liable to deteriorate on keeping than is eosin.

If, instead of adding methylenblue and eosin to the same liquid these dyes are dissolved separately in the concentrations given above and are placed in glass cells 3 cm. thick, one in front of the other, the transmitted light can then be compared with that of a solution of methylenblue-eosin of similar concentration contained in a third cell of the same dimensions as the former. Examined in this way in alcoholic solution the colour of methylenblue-eosin is found to be accurately matched, when the same concentrations are employed as in the test-tube experiment, if eosin in the form of the sodium salt is employed ; if, however, eosin in the form of the free acid is used, the colour of the apposed solutions, though approximating very nearly to that of the solution of methylenblue-eosin is not quite identical with it (Table 2, Exps. 2 and 4). When watery solutions are employed, the colour of the apposed liquids is too blue compared with that of the single liquid, whichever form of eosin is used, the same concentrations being employed as for the test-tube experiments above described ; if, however, the concentration of the eosin is sufficiently increased (Table 2, Exps. 7 and 8), then although a very slight increase or diminution of the concentration of the eosin causes the colour of the apposed cells to become too red or too blue, nevertheless an absolute identity of colour is not attainable. In this connection reference should be made to Table 1, in which an attempt is made to exhibit the relative colour of the dyes employed in a form suitable for ready comparison.

If the spectra of the apposed liquids are studied (Fig. 1), it is found that in all cases the spectra are the sum of those of the individual liquids, as is to be expected, when it is borne in mind that eosin has no absorptive effect whatever at the red end of the solar spectrum, nor does methylenblue absorb any light in the central part of the solar spectrum. It will now be readily understood that identity of colour is obtainable only when methylenblue-eosin is matched with methylenblue and water soluble eosin, alcohol being the solvent employed. The spectroscope thus affords a valuable means of checking and con-

firming the conclusions arrived at by the eye. The approximation to equality attained by increasing the strength of apposed watery solutions of eosin (Table 2, Exps. 7 and 8; cp. Fig. 1, D to F) is reached by increasing the absorption of light towards the solar line F, but no true equality of colour is possible.

Before proceeding to consider the bearing of the above data upon the nature of the staining act of methylenblue-eosin, it will be of advantage to make a brief reference to the hydrolysis of organic salts. Water, which possesses a very high dielectric constant and at the same time exhibits an extremely low degree of ionisation, has the property of hydrolysing salts formed by the combination of (1) a weak base with a strong acid, or (2) a weak acid with a strong base, or (3) a weak base with a weak acid. Thus anilin hydrochloride in ·03 N concentration is hydrolysed to the extent of 2·6%, while urea hydrochloride in the same concentration is hydrolysed to as much as ·95%[1]; potassium cyanide in ·02 N concentration is hydrolysed to the extent of 2·3%[2]; anilin acetate in ·01 N concentration is hydrolysed to the extent of 55%.[3] From these illustrations which can be multiplied it is obvious that, before attempting to explain the nature of methylenblue-eosin staining it is essential to ascertain how much this dye is hydrolysed by the solvent employed.

In solution in alcohol the spectroscopic characters of methylenblue-eosin show that this dye is not hydrolysed into methylenblue (free base) and eosin (free acid) to any recognisable extent, for the absorption spectrum of methylenblue-eosin in the green coincides with that of water soluble eosin, and no modification due to the presence of alcohol soluble eosin can be detected (Fig. 1, A to D). The question now arises, what is the smallest amount of alcohol soluble eosin, the addition of which (in an apposed cell) can be recognised spectroscopically. This addition may be put at about 25% of the concentration of the methylenblue-eosin examined ($2{\cdot}8 \times 10^{-5}$ N), when the spectroscopic appearance is observed, but when the colour

1. Shields (1893), *Zeitschr. f. physik. Chem.*, Bd. XII, s. 167; Walker (1899), *ibid.*, Bd. XXXII, s. 137.

2. Walker (1889), *Zeitschr. f. physik. Chem.*, Bd. IV, s. 319.

3. Arrhenius and Walker (1890), *Zeitschr. f. physik. Chem.*, Bd. V, s. 18.

is judged with the naked eye an addition of 10%, or under extremely favourable circumstances of $2\frac{1}{2}$%, can be recognised. If methylenblue (with or without the addition of an equivalent amount of potassium hydroxide), or alcohol soluble eosin is employed in alcoholic solution in one-tenth of the concentration in which methylenblue-eosin is ordinarily used (*i.e.* ·005 N or ·6%)[1] for staining purposes in alcohol, the respective blue and red differential staining cannot be obtained or is exceedingly faint ; to obtain staining equal to that of methylen-blue-eosin in the same period of time, the concentration must equal or, better still, exceed that of the methylenblue-eosin stain. Since, therefore the latter dye in alcoholic solution is not hydrolysed to an extent sufficient to furnish a possible explanation of its staining power, the conclusion follows that its staining act is a chemical reaction in which the dye molecules are broken up, and not a purely physical process. It is interesting to observe that no recognisable hydrolysis of water soluble eosin in $2 \cdot 5 \times 10^{-5}$ N concentration in alcohol is obtainable, for if sodium hydrate dissolved in absolute alcohol is added no change of colour or spectrum can be detected. Owing to the relatively low value of the dielectric constant of alcohol (26 for ethyl alcohol) compared with that of water (80) ionisation of the dyes need not be taken into account.

In water, methylenblue-eosin presents an absorption spectrum in the green (Fig. 1, H) differing slightly but distinctly from that of water soluble eosin (Fig 1, G), while its absorption spectrum in the red differs from that of methylenblue in the form of chloride (Fig. 1, E) or of free base (Fig. 2, *x*). Since the absorption spectrum of methylenblue-eosin is not compounded of that of methylenblue and water soluble eosin, colour matching by apposition is not possible as in the case of alcoholic solutions, and the only means of judging of the existence of hydrolysis is by means of spectroscopic examination. Unfortunately this, while failing to afford evidence of eosin in the form of free acid, is not sufficiently delicate (cp. p. 413) to afford proof that hydrolysis, if present, would be in so small a degree that the possibility

1. In methyl alcohol (Jenner's stain). In water the concentration is usually about one-fifteenth of this amount.

of the red and blue staining being a physical process would be altogether excluded. The colour and spectrum of methylenblue chloride in water, it may be observed, do not differ from those of the free base in water (Fig. 2, *x*). The spectrum of water soluble eosin in $2{\cdot}5 \times 10^{-5}$ N concentration in water affords no evidence of hydrolysis, for on adding sodium hydrate no change is produced ; in the stronger solution used for staining (·005 N) the degree of hydrolysis must be insignificant, since the degree of hydrolysis is inversely proportional to the square root of the concentration.[1] It does not, however, follow that the production of alcohol soluble eosin in watery solution of methylenblue-eosin would be relatively less in the ·005 N concentration used for staining than in the concentration ($2{\cdot}8 \times 10^{-5}$ N) submitted to spectroscopic examination, for in the case of a salt formed, as is methylenblue-eosin by the combination of a weak base with a weak acid, the amount of hydrolysis occurring may be independent of the concentration.[2] In such a case also a marked degree of ionisation may be present. Thus anilin acetate in watery solution is not only hydrolysed to about 55% in all concentrations, but the undissociated fraction is nearly completely ionised in centinormal concentration. Ionisation might be imagined to account in part for the circumstance that the spectrum of methylenblue-eosin is not compounded of those of methylenblue and eosin (sodium salt). This may be so, but the data given in the next section indicate that such an interpretation is insufficient to afford a satisfactory explanation of the difference in question. A more probable explanation is that the chromophore radicals in methylenblue-eosin mutually modify each other, though it is to be noted that no such modification is to

1. According to the formula $x = \sqrt{\frac{1}{c} \cdot \frac{k_w}{k_s}}$, where x is the degree of hydrolysis, c is the concentration, k_w is the water constant ($1{\cdot}2 \times 10^{-14}$), and k_s the dissociation constant of the free acid of eosin. This applies only if k_s is much larger than k_w. K_s is unknown, but since water soluble eosin in aqueous solution is not precipitated by carbonic acid, it follows that k_s is not less than the dissociation constant of the latter (3×10^{-7}), so that the formula holds good for eosin.

2. The relation is $\left(\frac{1-x}{x}\right)^2 = \frac{k_b k_s}{k_w}$, where x is the degree of hydrolysis, k_b the dissociation constant of the free base of methylenblue, k_s that of the free acid of eosin, and k_w the water constant.

be observed in alcoholic solution. The presence or absence of ionisation is not of much importance in deciding the problem at issue, for the possibility of a physical as opposed to a chemical process occurring in methylenblue-eosin staining turns upon the occurrence of hydrolysis. It is interesting to observe that the addition of chlorine ions (*i.e.* of 0·13 N NaCl) to an aqueous solution of methylenblue (6×10^{-5} N) the effect of which would be to diminish ionisation of methylenblue, causes the second faint band in the red to become more distinct.[1] When the concentration of sodium ions is increased by adding sodium iodide to water soluble eosin in aqueous (or alcoholic) solution, the colour and spectrum undergo no immediate change (subsequently a slow change occurs requiring several weeks for its full development).

To sum up we may say that methylenblue-eosin in alcoholic solution is not perceptibly hydrolysed, and that its staining act is therefore a chemical, not a physical, process. The data at present available, however, do not enable a conclusion to be arrived at as to the nature of the staining act in aqueous solution.

Although direct proof of the nature of methylenblue-eosin staining in aqueous solution is not available, nevertheless the fact that the staining act in alcoholic solution is a chemical reaction implies, though it does not prove, that the same holds for aqueous solution. The circumstance that the blue staining of nuclear protoplasm is effected not only by methylenblue-eosin in both alcoholic and aqueous solution, no matter whether in the form of chloride or of free base, is not easy to explain as a physical process due to adsorption or selective solubility of the dye, because the physical conditions in the four cases are dissimilar ; on the other hand on the theory of stochiometric reaction no such difficulty is experienced, the process being in each case the same. The same may be said of the staining of the oxyphile granules of leucocytes by methylenblue-eosin in alcoholic and in watery

1. In alcoholic solution of methylenblue the addition of $CaCl_2$ in 2×10^{-2} N concentration or of HCl in 4×10^{-2} N concentration does not alter the spectrum. In each of these cases advantage is taken of the relation $\frac{\text{concentration of anion} \times \text{concentration of kation}}{\text{concentration of undissociated salt}} = \text{constant}$. If the colourless ion is increased in amount the amount of coloured ion becomes reduced to insignificant dimensions and does not appreciably modify the colour of the undissociated dye.

solution, by alcohol soluble eosin in alcoholic solution, and by water soluble eosin in watery solution, all three dyes being in the same molecular concentration.

It is very desirable that the condition of the dyes employed should be further defined in respect of osmotic pressure and of ionisation. The latter cannot at present be determined with sufficient accuracy to be of much value owing to the impossibility of ascertaining with accuracy the fraction of the conductivity which is to be attributed to impurities in the form of electrolytes. Osmotic pressure will be dealt with in the next section, but before passing to this subject some further details respecting the colour and spectra of the dyes employed, in various conditions, will be given.

It has long been known that when an acid, mineral or organic, is added to an alcoholic solution of methylenblue-eosin the solution assumes the colour of methylenblue and on spectroscopic examination shows the spectrum of methylenblue (Fig. 1, A) unchanged while the eosin bands are more or less obliterated (as in Fig. 2, s, t), and when the mixed liquid is used for staining sections only the eosin colouration appears. If, instead of acid, an alkali is added, the solution turns reddish violet in colour and shows the eosin (Fig. 1, B) spectrum, while the methylenblue spectrum becomes modified (as in Fig. 2, v); on using the liquid for staining sections only blue staining is obtained. If this observation is performed in a test tube, 2×10^{-5} N will be found a convenient concentration for the dye, and the acid and alkali should be employed in 1×10^{-3} N concentration; if the latter are employed in 5×10^{-5} N concentration the acid changes the colour of the liquid, but no change is produced by the alkali. In aqueous solution, contrary to what would be expected, no change of colour occurs. Now the action of acid in alcoholic solution is obviously directed to the acid radical of methylenblue-eosin, for the addition of acid to methylenblue in solution does not alter the colour or spectrum of the solution. Similarly alkalies in alcoholic solution act upon the basic radical of methylenblue-eosin, for the addition of alkalies to the sodium salt of eosin produces no change of colour (only after prolonged boiling with concentrated alkali is a change of colour producible).

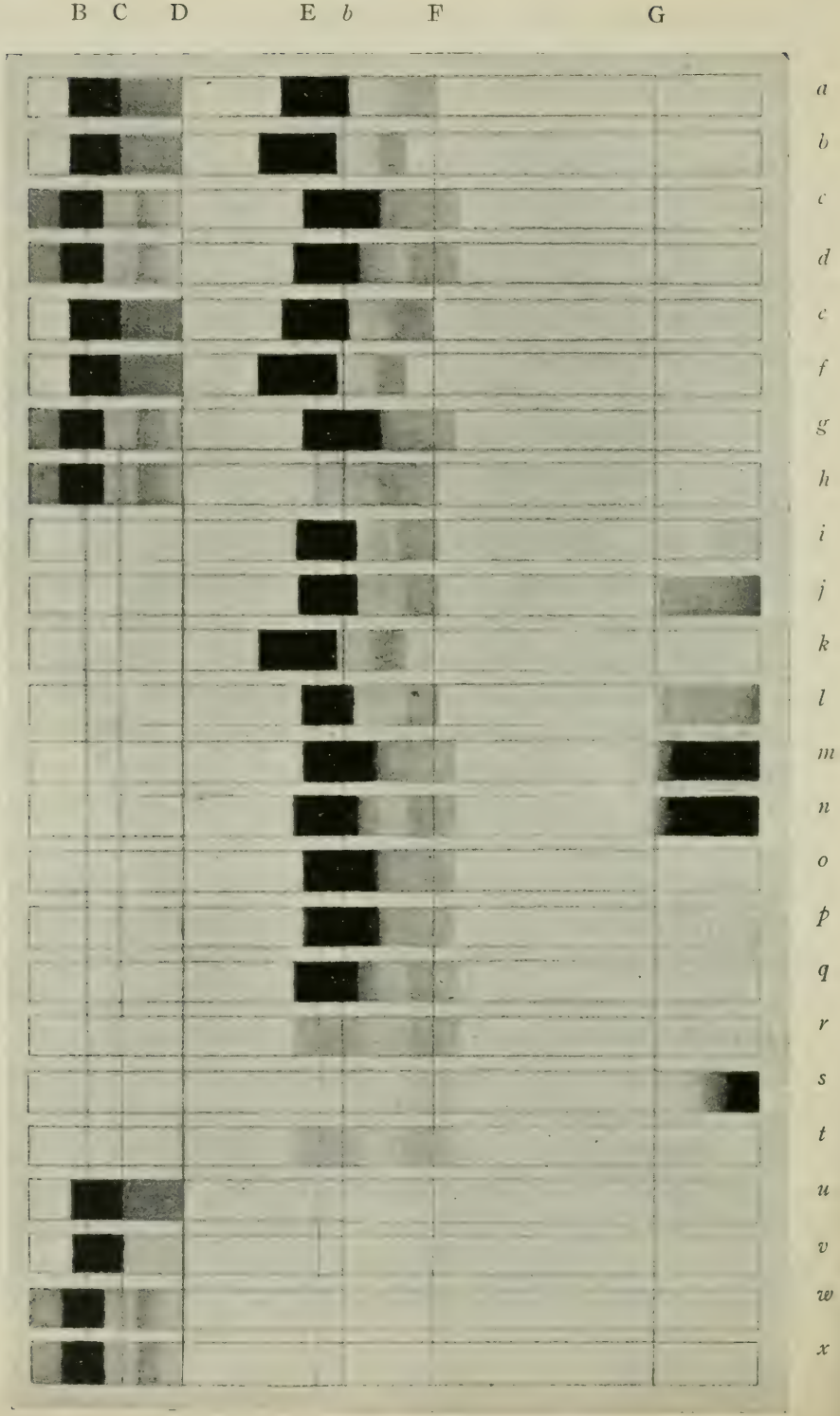

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Fig. 2. Spectra of various solutions viewed in a layer one centimetre thick, except when otherwise stated. The following spectra are each obtained from two liquids apposed in separate cells (Mb = methylenblue, Na_2E water soluble eosin, H_2E alcohol soluble eosin):

a.	Mb $2{\cdot}7 \times 10^{-5}$ N	+ Na_2E $2{\cdot}4 \times 10^{-5}$ N.	Solvent—	Absolute alcohol.
b.	,, 2·6 ,,	+ H_2E 2·5 ,,	,,	,,
c.	,, 2·7 ,,	+ Na_2E 2·4 ,,	,,	Water.
d.	,, 2·6 ,,	+ H_2E 2·5 ,,	,,	,,

The remaining spectra are each obtained from a single solution:

e.	Mb $2{\cdot}7 \times 10^{-5}$ N	+ Na_2E $2{\cdot}4 \times 10^{-5}$ N.	Solvent—	Absolute alcohol.
f.	,, 2·6 ,,	+ H_2E 2·5 ,,	,,	,,
g.	,, 2·7 ,,	+ Na_2E 2·4 ,,	,,	Water.
h.	,, 2·6 ,,	+ H_2E 3·0 ,,	,,	,,
i.	Na_2E 2·5 ,,	+ Na I ·7 N.	,,	Absolute alcohol.
j.	,, ,, ,,	+ Na I ·7 N.	,,	,,
k.	H_2E 2·5 ,,	+ Na I ·7 N.	,,	,,
l.	,, ,, ,,	+ Na I ·7 N.	,,	,,[1]
m.	Na_2E 2·3 ,,	+ Na I ·1 N.	,,	Water.[1]
n.	H_2E ,, ,,	+ Na I ·1 N.	,,	,,[1]
o.	Na_2E ,, ,,		,,	,,
p.	,, ,, ,,	+ NaCl 1·5 N.	,,	,,
q.	H_2E ,, ,,		,,	,,
r.	,, ,, ,,	+ NaCl 1·5 N.	,,	,,
s.	,, ,, ,,	+ H_2SO^4 1×10^{-3}	,,	Absolute alcohol.[2]
t.	,, ,, ,,	+ H_2SO_4 ,,	,,	Water.[2]
u.	Mb 2·7 ,,		,,	Absolute alcohol.
v.	,, ,, ,,	+ KOH 1×10^{-3}	,,	,,
w.	,, ,, ,,		,,	Water.
x.	,, ,, ,,	+ KOH 1×10^{-3}	,,	,,

1. Spectrum observed after the liquid had been mixed for a week.
2. In a layer three centimetres thick.

If to an alcoholic solution of methylenblue (the most convenient concentration being that of 1×10^{-4} N to 1×10^{-5} N) sodium or potassium hydrate is added in the proportion of one molecule to one of methylene-blue, the liquid preserves its colour and shows no alteration of its spectrum; if alkali is added in greater quantity, *e.g.* in the proportion of twenty molecules to one of methylenblue, the colour quickly changes to reddish-purple subsequently becoming purple-red, the methylenblue band in the spectrum showing less absorptive power between C and D while the dark band retains its position and intensity

undiminished, as in Fig. 2, *v*. In watery solution no change is produced either in colour or spectrum by the addition of alkali.

If to a dilute alcoholic or watery solution of eosin in the form of the sodium salt hydrochloric or sulphuric acid is added in equimolecular amount, the colour of the liquid changes and inclines to that of the acid eosin, the spectrum changing also to that of acid eosin, but the colours and spectrum are not quite pure for the further change next to be described is easily produced if too much acid is added.

Excess of acid (five to twenty molecules of acid to one molecule of eosin) almost completely decolourises eosin solutions of $2{\cdot}5 \times 10^{-5}$ N concentration, producing in alcohol a faint light brownish colour with an exceedingly faint fluorescence and the spectrum shown in Fig. 2, *s*., while in water a light reddish-brown colour with a very slight olive-green fluorescence is seen, and the faint absorption spectrum, shown in Fig. 2, *t*., is obtained. To observe these spectra a layer of fluid three or more centimetres in thickness is necessary.

If to a $2{\cdot}5 \times 10^{-5}$ N solution of water soluble eosin sodium iodide in ·1 N or greater concentration is added, the colour in alcoholic solution changes slowly to a slightly brownish pink and the spectral bands at E and F are slightly displaced towards the blue end, while a faint band appears beyond G; at the end of a week the solution has become of a darker yellowish brown colour and the latter band is deeper (Fig. 2, *i* and *j*). In water, the dye, after the addition of iodide of sodium retains its colour unchanged but loses its fluorescence, the bands at E and F being unaltered, but a dark band has appeared extending to the right of G.

If alcohol soluble eosin is employed, the addition of sodium iodide produces in alcoholic solution a slightly brighter and more violet-pink colouration, but no recognisable alteration of the spectrum is at first noted; at the end of a week a yellowish-brown colour is produced, and the spectrum is that shown in Fig. 2, *l*. In watery solution the colour does not change on adding sodium iodide, but fluorescence is abolished, the spectrum remaining unchanged; at the end of a week the liquid has become of a reddish-brown colour and exhibits the spectrum shown in Fig. 2, *n*.

If to an aqueous solution of water soluble eosin, sodium chloride in 0·1 N concentration is added, no change in colour or spectrum occurs (Fig. 2, *p*). If eosin in the form of free acid is employed the colour becomes of a lighter and purer violet pink and the absorption bands become lighter, without, however, changing their position (Fig. 2, *r*).

Osmotic Pressure

Further data are necessary to elucidate the nature of the staining act, which forms the subject of this investigation. In particular it is desirable to ascertain the osmotic pressure exerted by the dyes. Observations of this kind made with aniline dyes appear to be limited to some determinations made by Krafft,[1] who found that the molecular weight of perfectly dry rosaniline chloride, methylenblue and methylviolet, ascertained by observing the raising of the boiling-point of absolute ethyl alcohol, corresponded to the accepted formulae of these dyes, but that when care was not taken to exclude the presence of traces of water, or when water was used as the solvent, the raising of the boiling-point was less than that required by theory and an apparent molecular weight, perhaps twice as large, was obtained. The latter observation is of considerable significance, for, taken in conjunction with the well known property which such dyes exhibit to separate out from their solvents not in the crystalline, but in the amorphous or globomorphous state, it indicates that these substances are in the latter case present in the colloidal form. Whether the dye is present in an imperfectly developed colloidal state still capable of exerting a definite osmotic pressure, or whether it is present in two different states, being partly in colloidal, and partly in true, solution, is not yet determined, but the latter view is probably correct since it is found that, when the dye is added in successive amounts to the solvent, the raising of the boiling point or lowering of the freezing point is most marked after the first addition, and each subsequent addition of the dye is attended with less effect than the preceding

1. 'Ueber colloïdale Salze als Membranbildner beim Färbeprocess,' *Ber. d. deut. chem. Gesellschaft*, 1899, 32 Jahrg., Bd. II, S. 1608.

It is difficult to explain this circumstance except on the assumption that the first portion added passes largely into true solution, the solvent becoming rapidly saturated by the quantities of the dye subsequently added, so that the only form in which further solution can take place is the colloidal form. It is interesting to note that Krafft found that fuchsin, methylenblue and methylviolet diffused through parchment paper, while benzopurpurin, benzazurin and azoblue showed no trace of diffusion.

In Tables 3 and 4 some determinations[1] are given of the influence of methylenblue-eosin, methylenblue and alcohol soluble eosin upon the boiling point of methyl alcohol, and that of methylenblue and water soluble eosin upon the freezing point of water. In these determinations no attempt was made to obtain the dyes in a perfectly dry condition,[2] since it was desired to investigate their condition under circumstances similar to those obtaining in the staining liquids, alcoholic and watery, used in histological technique, in the preparation of which no attempt is made to exclude the presence of traces of water from the dyes employed.

The results obtained in Tables 3 and 4 show that methylenblue-eosin, as also methylenblue and both forms of eosin, dissolved in methyl alcohol or water, exist in the colloidal state, thus resembling fuchsin, methylviolet, tannin and soap. The last column of these Tables gives the amount of dye which may be regarded as present in true solution, calculated on the assumption that the dyes exist in a diphasic condition. These results are, however, in all probability, except in the case of Exp. 6, Table 3, too high owing to the difficulty of completely removing from the dyes slight impurities, which may exert osmotic pressure. The disturbing influence of even mere traces of such impurities is readily understood, when it is borne in mind that a 0·01 N concentration of methylenblue-eosin, for example,

1. With Beckmann's apparatus, *Zeitschr. f. physikal. Chemie*, 1902, Bd. XL, S. 129.

2. The dyes employed contained 7% to 12% of water. If methylenblue-eosin is dried to constant weight at 100° C., it becomes very slightly soluble in alcohol, requiring repeated renewal of the solvent in order to obtain complete solution; the spectrum of the dye is not altered. The same is true of alcohol soluble eosin. In neither case is the solubility improved by soaking the dried dye again in water. On the other hand pastilles of the freshly precipitated dye, incompletely dried in air, dissolve at once in methyl alcohol.

would be represented by a 1·2% solution, while the same concentration of sodium chloride would be represented by a ·06% solution. Alcohol soluble eosin can readily be obtained in a pure condition by adding an equivalent amount of sulphuric acid to water soluble eosin in aqueous solution; Exp. 6, Table 3, was made with eosin so precipitated,[1] and subsequently thoroughly washed and incompletely dried in air, while Exp. 5, Table 3, was made with a sample obtained from Grübler. The purification of the remaining dyes, in particular the separation of small quantities of salt, is attended with great difficulty. Sodium chloride, for example, is formed when methylenblue and water soluble eosin are mixed in the required proportion; if the bulky precipitate thus formed is washed with large quantities of distilled water serious loss from solution of the dye, which is highly insoluble only in saline liquids, occurs. In Exp. 3, Table 4, a sample of eosin puriss.[2] was employed. In Exp. 4, the dye was prepared by dissolving freshly precipitated and carefully washed alcohol soluble eosin in the calculated amount of a solution of pure sodium hydrate and evaporating to dryness[3]; a lower degree of osmotic pressure was noted. The methylenblue employed was supplied by Grübler in a specially purified form suitable for injection *intra vitam*. The sparing solubility of methylenblue-eosin in alcohol renders difficult accurate ebullioscopic observations, the degree of alteration of the boiling point obtained being inconveniently small; with water as solvent no such observations are possible.[4]

The difficulty of ensuring the absence of traces of electrolytes from the dyes investigated renders determinations of the conductivity of these solutions of little value in affording knowledge of the degree of ionisation present.

1. For analysis, see p. 409.

2. For analyses of these dyes see pp. 407-411.

3. Commercial water soluble eosin may exhibit more than twice the osmotic pressure required by theory for the pure salt.

4. It is interesting to note that Michaelis (Dent. medizin. Wochenschr., 1904, No. 42; Virchow's Archiv, 1905, Bd. 179, S. 195) finds that watery solutions of eosin and methylenblue, though optically inhomogeneous, are not resolvable into submicroscopic particles. Michaelis and also Zsigmondy (Zur Erkenntniss der Kolloide, 1905, S. 160) found, however, that a watery solution of fuchsin was partially resolvable into ultramicroscopic particles, whence both observers conclude that this dye is diphasic in such solution.

Although the osmotic pressure of methylenblue-eosin in aqueous solution cannot be determined, the marked tendency of such solutions to form films and amorphous precipitates of the dye, especially on the addition of a trace of neutral salt, such as sodium chloride, shows the highly colloidal nature of such solutions. Alcohol soluble eosin in aqueous solution can be equally readily precipitated by neutral salts ; methylenblue is similarly precipitated, but stronger concentration of salt is required. Water soluble eosin is not precipitated by sodium chloride or iodide.

It appears, therefore, that all the stains dealt with in this paper exhibit colloidal characters in alcoholic and aqueous solutions.

The occurrence of the colloidal state probably explains why it is that no recognisable degree of hydrolysis could be recognised by the method of observation adopted in the preceding section. It would appear that in the colloidal state the dyes in question are shielded from the dissociative influence of the solvent.

Summary

The main conclusions resulting from the present investigation are the following :—

1. The staining act of methylenblue-eosin in alcoholic solution is a chemical reaction.
2. Methylenblue-eosin in alcoholic and aqueous solution exhibits colloidal characters, as do also methylenblue and eosin in the water soluble and alcohol soluble forms.

Table I. Colour and Fluorescence of Dyes employed

Dye	Concentration	Colour by Transmitted Light: In Alcohol	Colour by Transmitted Light: In Water	Fluorescence: In Alcohol	Fluorescence: In Water
Methylenblue, $2C_{16}H_{18}N_3SCl + ZnCl_2 + H_2O$	·001 N	Dark blue	Dark blue	None	None
,, ,, ,,	·00001 N	Blue	Blue	None	None
Methylenblue, to which potassium hydrate has been added[1]	·001 N	Dark purple blue	Dark blue	None	None
,, ,, ,,	·00001 N	Purple blue	Blue	None	None
Water soluble eosin, $C_{20}H_6Br_4O_5Na_2$ ..	·001 N	Yellowish red	Brownish red	Strong light yellowish green	Slight olive green
,, ,, ,, ..	·00001 N	Bright pink	Slightly brownish pink	Strong light yellowish green	,, ,,
Alcohol soluble eosin, $C_{20}H_8Br_4O_5$	·0003 N[2]	Deep pink	Deeper brownish pink	Slight yellowish green	,, ,,
,, ,, ,, ...	·00001 N	Brilliant slightly violet pink	Violet pink with slight brownish tint	Very slight yellowish green	Very slight olive green
Methylenblue-eosin, $C_{52}H_{40}N_6S_2Br_4O_5$ ..	·001 N	Dark purple	——	Strong dark yellowish green	——
,, ,, ,, ,, ..	·00001 N	Bluish purple	Reddish purple	Strong dark yellowish green	Very slight dark olive green

1. In the proportion of six molecules of potassium hydrate to one molecule of methylenblue.
2. In ·001N concentration, alcohol soluble eosin is of a darker red colour than water soluble eosin in the same concentration.

Table II. Determination of the Amounts of Methylenblue and of Eosin required to Produce Equality of Colour with a Solution of Methylenblue-Eosin

No. of Exp.	Concentration of: Methylenblue-eosin	Concentration of: Methylenblue	Concentration of: Eosin	Solvent	Mode of Observation	Result	Remarks
1	$\cdot 56 \times 10^{-5}$ N	$\cdot 54 \times 10^{-5}$ N	Water sol. $\cdot 48 \times 10^{-5}$ N	Alcohol	Methylenblue-eosin placed in one test-tube; methylenblue and eosin put in a second test-tube of the same diameter as the first	Complete equality of colour	Cp. spectra A, B and D, fig. 1[1]
2	,,	·53 ,,	Alc. sol. ·50 ,,	,,		Very nearly complete equality of colour	Cp. spectrum C, fig. 1[1]
3	,,	·54 ,,	Water sol. ·48 ,,	,,	Methylenblue-eosin, methylenblue and eosin placed separately in glass cells, the two latter being apposed and comparison made with the first	Complete equality of colour	
4	,,	·53 ,,	Alc. sol. ·50 ,,	,,		Very nearly complete equality of colour	
5	,,	·54 ,,	Water sol. ·48 ,,	Water	In test-tubes, as in 1 and 2	Complete equality of colour	Cp. spectra E, F and H, fig. 1[1]
6	,,	·53 ,,	Alc. sol. ·60 ,,	,,	,, ,,	Very nearly complete equality of colour immediately after mixing	Cp. spectrum G, fig. 1[1]
7	,,	·54 ,,	Water sol. .48 ,,	,,	In glass cells, as in 3 and 4	Methylenblue + eosin: too blue	
7	,,	·54 ,,	,, ·82 ,,	,,	,, ,,	Nearly complete equality of colour	
8	..	·53 ,,	Alc. sol. ·50 ,,	,,	,, ,,	Methylenblue + eosin: too blue	
8	,,	·53 ,,	,, ·77 ,,	,,	,, ,,	Nearly complete equa-	

TABLE III. RAISING OF THE BOILING POINT OF METHYL ALCOHOL, BROUGHT ABOUT BY METHYLENBLUE-EOSIN, METHYLENBLUE AND ALCOHOL SOLUBLE EOSIN. AMOUNT OF SOLVENT EMPLOYED 15·78 g. (MOLECULAR RAISING OF B.P. = 8·8° C.).

No. of Experiment	DYE	Amount of Dye employed	Molecular Concentration of Dye	Observed Raising of Boiling Point	Corresponding Molecular Concentration (calculated)	Observed Raising of Boiling Point in Percentage of Calculated Amount
1	Methylenblue-eosin, 1st sample...	0·098 g.	0·0052 N.	+ 0·010° C.	0·0011 N.	22
2	„ „ 2nd „ ...	0·100 g.	0·0053 N.	+ 0·030° C.	0·0037 N.	71
3	Methylenblue	0·352 g.	0·0283 N.	+ 0·091° C.	0·0104 N.	37
4	„	0·101 g.	0·0081 N.	+ 0·013° C.	0·0015 N.	18
5	Eosin (alcohol soluble) 1st sample	0·154 g.	0·0152 N.	+ 0·016° C.	0·0018 N.	12
6	„ „ 2nd „	0·146 g.	0·0144 N.	+ 0·000° C.	——	0

TABLE IV. LOWERING OF THE FREEZING POINT OF WATER, BROUGHT ABOUT BY METHYLENBLUE AND WATER SOLUBLE EOSIN (SODIUM SALT). AMOUNT OF SOLVENT EMPLOYED 13 g. (MOLECULAR LOWERING OF F.P. = 19° C.).

No. of Experiment	DYE	Amount of Dye employed	Molecular Concentration of Dye	Observed Lowering of Freezing Point	Corresponding Molecular Concentration (calculated)	Observed Lowering of Freezing Point in Percentage of Calculated Amount
1	Methylenblue	0·358 g.	0·035 N.	– 0·102° C.	0·0054 N.	15
2	„	0·031 g.	0·003 N.	– 0·010° C.	0·0005 N.	19
3	Eosin (water soluble) 1st sample	0·447 g.	0·050 N.	– 0·781° C.	0·0412 N.	82
4	„ „ 2nd „	0·143 g.	0·016 N.	– 0·106° C.	0·0050 N.	31

Die Reaktionsgeschwindigkeit zwischen Opsonin und der roten Blutzelle.

Von

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Die Wirkung der Opsonine auf rote Blutkörperchen ist einer der im Tierkörper sich abspielenden Vorgänge, die in den letzten Jahren die Aufmerksamkeit auf sich gezogen haben, und deren Besonderheit gerade darin liegt, dass sie für den Organismus ein Verteidigungsmittel gegen verletzende Einflüsse darstellen. Als Beispiel für solche Vorgänge mögen neben den schon genannten die Wirkung von Antitoxin auf Toxin, von Hämolysinen und Agglutininen auf rote Blutkörperchen, von Präzipitinen auf gelöste Proteïde und von Opsoninen und Lysinen auf Bakterien erwähnt werden. Die an derartigen Vorgängen beteiligten Substanzen lassen sich in reinem Zustande nicht isolieren; ihre Natur ist ungewiss: einige sind, da thermolabil, vermutlich koagulierbare Proteïde; andere werden, da sie nicht durch Gelatine oder Pergamentpapier oder nur sehr schwer in die erstere gehen, für kolloidal gehalten. Ob die Substanzen alle wirklich einheitlich sind, ist gleichfalls ungewiss; in einzelnen Fällen ist dies sogar zweifelhaft.

Alle Versuche, die Natur der Interaktion von Substanzen dieser Art zu studieren, sind mit Schwierigkeiten verbunden; besonders da es keine hinreichend exakten quantitativen Methoden gibt, mit Hilfe deren man die Menge der gegeneinander wirkenden Substanzen bestimmen kann, ist es schwer festzustellen: 1. ob die Interaktion in festen Verhältnissen wie in chemischen Reaktionen stattfindet oder in wechselnden Verhältnissen, die von der jeweiligen Konzentration wie in kolloidalen Reaktionen abhängen; 2. nach welcher Geschwindigkeit die in Rede stehende Interaktion verläuft. In einigen Fällen bildet die Unbeständigkeit bei der Erhaltung ein weiteres Hindernis für die Untersuchung.

Die Interaktion von roten Blutkörperchen und ihren entsprechenden Opsoninen, die den Gegenstand dieser Untersuchung bildet, ist ein

etwas einfacheres Problem, da sich die Bestimmung des Betrages der zur Verwendung kommenden roten Blutkörperchen leicht machen lässt, und sich die Opsoninbestimmung in den Versuchen ebenfalls leicht durchführen liess[1]). Die letztere Bestimmung (vergl. Tabelle 1) beruht auf der Tatsache, dass, wenn man rote Blutkörperchen und ihre entsprechenden Opsonine zusammenbringt, und wenn die letztern in dem Minimalbetrage vorhanden sind, der erforderlich ist zur Hervorrufung derjenigen Sensibilisation der erstern, welche zur Herbeiführung einer völligen Phagocytose nötig ist[2]), der Grad der Verdünnung des Opsonins keinen irgend erkennbaren Einfluss auf den Grad der Sensibilisation der roten Blutkörperchen ausübt[3]).

Obwohl die von mir angestellten Versuche nicht bis zur Lösung der Frage geführt haben, ob die Verbindung von roter Blutzelle und Opsonin in festen oder in wechselnden Verhältnissen stattfindet, so hat sich doch die Möglichkeit herausgestellt, die Geschwindigkeit, mit welcher diese Verbindung vor sich geht, zu untersuchen. In drei Beobachtungsreihen, deren Versuchseinzelheiten anderswo dargelegt sind[4]), gelangte ich, wenn ich rote Blutkörperchen in dem opsoninhaltigen Serum für wechselnde Zeiträume beliess, zu folgenden Ergebnissen:

[1]) Die Existenz von Opsoninen wurde zuerst in bezug auf Bakterien von Wright und Douglas erkannt (An experimental investigation of the role of the body fluids in connection with Phagocytosis: Proc. of the Roy. Soc. **72**, 357 (1903); **73** (1904), von denen der Name Opsonin herrührt. Diese Substanzen wurden weiter untersucht von Bullock und Atkin (Experiments on the nature of the opsonic action of the blood serum: Proc of the Roy. Soc. **74**, 379. 1905) und von Bullock und Western (The specificity of the opsonic substances in the blood serum: Proc. of the Roy. Soc. **B. 77**, 531. 1906). Die Beziehung von Opsoninen zu roten Blutzellen hat zuerst Barratt beobachtet (The phagocytosis of red blood corpuscles: Proc. of the Roy. Soc. **76**, 524. (1905).

[2]) Barratt, On opsonins in relation to red blood cells: Proc. of the Roy. Soc. **B. 79**, (1907). In dieser Arbeit ist die angewandte Methode angegeben.

[3]) Die Bestimmung von Erythrocytenopsoninen lässt sich leicht zufriedenstellend ausführen, wenn die „spontane" Phagocytose vermieden wird. Dies erreicht man, wenn man die roten Blutzellen (vorher mit Opsonin sensibilisiert), Leukocyten und das Serum, in welchem die Leukocyten gehalten, alle von derselben Tierart stammen. Der Eintritt der spontanen Phagocytose tritt der Bestimmung von Bakterienopsoninen, wie auch der von Erythrocytenopsoninen entgegen, wenn den roten Blutzellen fremde Leukocyten angewendet werden. Erythrocytenopsonine sind beständige Substanzen.

[4]) Loc. cit. Bei diesen Versuchen befand sich das Opsonin im Serum eines Kaninchens, welchem rote Blutzellen des Meerschweinchens eingespritzt worden waren. Ebensolche rote Blutzellen werden zur Ausziehung des Opsonins benutzt. Die Leukocyten waren einem Meerschweinchen entnommen und wurden im Meerschweinchenserum aufbewahrt.

Tabelle 1.

Ausziehung von Opsonin aus Serum mittels roter Blutkörperchen. Nach Ablauf der verzeichneten Zeiträume wurden die roten Blutkörperchen durch Zentrifugieren entfernt, und es wurde die zurückbleibende Opsoninmenge bestimmt; die ursprünglich vorhandene Menge ist = 100 gesetzt. Temperatur 37°.

Versuchsserie	Zeitraum der Deopsination	Vorhandene Opsoninmenge	
A.	t_0 bei Beginn des Versuchs	c_0	100
	t_1 nach 5 Minuten	c_1	4
	t_2 „ 20 „	c_2	2
	t_3 „ 80 „	c_3	1
B.	t_0 bei Beginn des Versuchs	c_0	100
	t_1 nach 5 Minuten	c_1	15
	t_2 „ 20 „	c_2	4
	t_3 „ 80 „	c_3	1·5
C.	t_0 bei Beginn des Versuchs	c_0	100
	t_1 nach 5 Minuten	c_1	12
	t_2 „ 40 „	c_2	2

Wenn man zwischen diesen Resultaten und den in mono-, di- und trimolekularen Reaktionen, wie sie sich in wahren Lösungen abspielen, einen Vergleich anstellt, indem man in die Formel für die Werte der Geschwindigkeitskonstante k in solchen Reaktionen die Masse der Zeit (t) und Konzentration (C) aus Tabelle 1 einsetzt, zeigt sich (Tabelle 2), dass man sehr verschiedene Werte für k nach der ersten und dritten Formel erhält, während sich eine engere Beziehung bei der zweiten Formel einstellt. Mit andern Worten: Die Interaktion von Opsonin und roter Blutzelle geht, soweit die angewandten Methoden die Anstellung eines Vergleichs erlauben, mit einer der dimolekularen Reaktion ähnlichen Geschwindigkeit vor sich. Was auch die Natur des Opsonins und des wirksamen Bestandteils der roten Blutzelle sei, so kann sie offenbar nicht derart sein, dass die Interaktion der beiden mit einer Geschwindigkeit wie eine monomolekulare Reaktion in einem homogenen System verliefe.

Die Interaktion zwischen roten Blutzellen und Opsonin findet hingegen in einem heterogenen System statt, und die Formeln, welche die Reaktionsvorgänge im homogenen System darstellen, können, da sie von der Massenwirkung abhängen, nicht ohne weiteres auf die heterogenen Systeme angewendet werden. Das einzige bis jetzt bezüglich der Reaktionsgeschwindigkeit mit einiger Vollständigkeit untersuchte heterogene System scheint das zu sein (Tabelle 2), wo ein fester Körper in eine Flüssigkeit getaucht wird, die eine lösende Wirkung auf ihn aus-

Tabelle 2.

Werte von k aus den in Tabelle 1 angegebenen Daten berechnet.

Zeitraum	$\frac{1}{t''-t'} \log \frac{c'}{c''}$	$\frac{1}{t''-t'}\left(\frac{1}{c''}-\frac{1}{c'}\right)$	$\frac{1}{2[t''-t']}\left(\frac{1}{[c'']^2}-\frac{1}{[c']^2}\right)$
$t_1 - t_0$ Serie A.	0·219	0·025	0·0017
$t_2 - t_0$ „	0·085	0·025	0·0125
$t_3 - t_0$ „	0·025	0·012	0·0063
$t_2 - t_1$ „	0·020	0·017	0·0125
$t_3 - t_1$ „	0·012	0·012	0·0062
$t_4 - t_2$ „	0·005	0·008	0·0125
$t_1 - t_0$ Serie B.	0·165	0·011	0·0005
$t_2 - t_0$ „	0·699	0·012	0·0016
$t_3 - t_0$ „	0·022	0·008	0·0028
$t_2 - t_1$ „	0·028	0·012	0·0020
$t_3 - t_1$ „	0·013	0·008	0·0030
$t_3 - t_2$ „	0·007	0·007	0·0032
$t_1 - t_0$ Serie C.	0·186	0·025	0·0069
$t_2 - t_0$ „	0·425	0·012	0 0033
$t_2 - t_1$ „	0·020	0·012	0·0035

übt. Nernst[1]) hat nachgewiesen, dass der sich abspielende Prozess sich in drei Stadien zerlegen lässt: 1. Übergang vom festen in den flüssigen Zustand; 2. chemischer Vorgang in einem der beiden obigen Zustände; 3. Diffusion entweder des gelösten Materials von der Berührungsfläche aus in die umgebende Flüssigkeit oder der die Lösung hervorrufenden Substanz von der Flüssigkeit aus gegen die Berührungsfläche. Das erste Stadium verläuft mit so grosser relativer Geschwindigkeit, dass die dabei verstreichende Zeit im Vergleich zu der für die übrigen Stadien erforderlichen vernachlässigt werden. Wenn der zweite Prozess sehr langsam ist im Vergleich zu dem dritten (Diffusion), so wird die Reaktionsgeschwindigkeit gewöhnlich unabhängig von der Diffusion und abhängig von der chemischen Wirkung. Dies tritt ein, wenn Arsentrioxyd im Wasser[2]) gelöst wird, oder wenn Ester in Benzol in saurer Lösung verseift werden[3]). In diesem Falle ist das Massenwirkungsgesetz anwendbar. Wenn dagegen die Geschwindigkeit der chemischen Veränderung im Vergleich zu der Diffusion rasch ist, so wird die Reaktionsgeschwindigkeit abhängen von der Differenz zwischen der

[1]) Theorie der Reaktionsgeschwindigkeit in heterogenen Systemen: Diese Zeitschrift **47**, 52 (1904).

[2]) Drucker, Zwei Fälle von Katalyse im heterogenen Systeme: Diese Zeitschr. **36**, 201 (1901). — Zur Geschwindigkeit und Katalyse im heterogenen Systeme: Diese Zeitschr. **36**, 693 (1901).

[3]) Goldschmidt, Die Reaktionsgeschwindigkeit in heterogenen Systemen: Diese Zeitschr. **31**, 235 (1899).

Sättigungskonzentration (C) des festen Körpers in der Lösung und der (c) tatsächlich gerade unmittelbar in Berührung mit der festen Substanz stehenden Flüssigkeitsschicht; dann verläuft die Reaktion nach der Formel von Noyes und Whitney[1]):

$$\frac{dx}{dt} = A(C - c)$$

wo dx den in der Zeit dt gelösten Betrag angibt, und A eine Konstante ist, welche aus der Formel:

$$A = \frac{v}{t} \log \frac{C}{C - c}$$

berechnet werden kann, wo v das „Aussenvolumen" ist. Diese Formel ist als gültig nachgewiesen für die Lösung von Benzoesäure und Bleichlorid in Wasser. Bruner und Tolloczko[2]) zeigten, dass die Oberflächengestaltung des festen Körpers keinen Einfluss auf die Auflösungsgeschwindigkeit hat, während Brunner[3]) eine weitere Bestätigung bezüglich der Lösung von Hydroxyden, Metallen und Carbonaten in Säuren erbrachte, wofür die Beziehung:

$$-\frac{dx}{dt} = \frac{DF}{\delta} \cdot c$$

besteht. In diesem Falle gilt das Massenwirkungsgesetz nicht.

Was nun die rote Blutzelle und Opsonin anlangt, so ist die Natur der wirkenden Substanzen unbekannt. Bekannt ist nur, dass der mit Opsonin in Wirkung tretende Bestandteil der roten Blutzelle sich nicht aus der roten Blutzelle durch längere Berührung mit 0·85%iger Kochsalzlösung ausziehen lässt: er muss daher ein Kolloid sein oder ein Kristalloid, dessen Austreten wohl dadurch verhindert wird, dass das äussere Gerüstwerk der roten Blutzelle als eine undurchlässige Membran wirkt. Man weiss nicht, ob Opsonin ein Kristalloid oder ein Kolloid ist. Es sind mithin die vier in Tabelle 3 aufgeführten Fälle in Betracht zu ziehen.

Wenn die Wirkung von roten Blutzellen auf Opsonin ein chemischer Vorgang ist, so würde sich Fall 4 auf Tabelle 3 ausschliessen, da Opsonin, seine Existenz unter diesen Bedingungen vorausgesetzt, unfähig sein würde, durch das kolloidale äussere Gerüstwerk der roten

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[1]) Über die Auflösungsgeschwindigkeit von festen Stoffen in ihren eigenen Lösungen: Diese Zeitschr. **23**, 689 (1897).

[2]) Über die Auflösungsgeschwindigkeit fester Körper: Diese Zeitschr. **35**, 283 (1900).

[3]) Reaktionsgeschwindigkeit in heterogenen Systemen: Dissert. Göttingen 1903.

Blutzelle zu treten[1]). In Fall 1, Tabelle 3 könnte die Interaktionsgeschwindigkeit abhängen entweder von der Diffusionsgeschwindigkeit von Opsonin, wenn diese relativ langsam wäre, oder von der chemischen

Tabelle 3.

Faktoren, welche die Reaktionsgeschwindigkeit von roten Blutkörperchen und Opsonin bestimmen, unter der Annahme, dass eine chemische Veränderung stattfindet.

Nr.	Natur des wirksamen Bestandteils im roten Blutkörperchen	Natur des Opsonins	Reaktionsgeschwindigkeit abhängig von		Art der Reaktion	
			a.	b.	a.	b.
1.	Kolloid	Kristalloid	(Diffusion)	Chemische Wirkung	(monomolekular)	(monomolekular oder) bimolekular
2.	„	Kolloid		? Konzentration		(? monomolekular) ? bimolekular
3.	Kristalloid	Kristalloid	(Diffusion)	Chemische Wirkung		(mono- oder) bimolekular
4.	„	Kolloid		—	—	—

Reaktionsgeschwindigkeit zwischen roten Blutzellen und Opsonin, wenn die Diffusion relativ schnell wäre, oder beide Prozesse könnten zusammen stattfinden; die erstere würde mit einer monomolekularen Reaktionsgeschwindigkeit sich abspielen, die zweite könnte mit einer monomolekularen oder einer bimolekularen Geschwindigkeit eintreten. Durch die in Tabelle 1 erhaltenen Ergebnisse wird jedoch eine monomolekulare Geschwindigkeit ausgeschlossen, so dass die zu der letztern führenden Bedingungen nicht aufrecht erhalten werden können und daher in Tabelle 3 in Klammern gesetzt sind.

Der zweite Fall (2. in Tabelle 3) weist erhebliche Schwierigkeiten auf, denn die Untersuchung der Frage, ob ein Kolloid eine chemische Wirkung auf ein Kolloid ausüben kann, ist noch nicht beantwortet. Wenn eine solche Wirkung möglich ist, so würde es prima facie nicht unwahrscheinlich sein, dass die Reaktion von der mechanischen Beimischung kolloidaler Teilchen abhinge und so abhängig wäre von der Konzentration der roten Blutzellen und Opsonin. Die Hilfsmittel zur Sicherung mechanische Beimischung dürften ebenfalls die Reaktionsgeschwindigkeit beeinflussen. Sowohl eine monomolekulare als auch eine dimolekulare Geschwindigkeit würde zulässig erscheinen, aber die erstere wird durch die in Tabelle 2 beobachteten Daten ausgeschlossen.

[1]) Wenn Opsonin ein diffusibles Kolloid wäre, so würde es eine Mischreaktion veranlassen, wobei beide Verhältnisse von 1 und 2 oder von 3 und 4 in Tabelle 3 obwalten würden.

Tabelle 4.

Faktoren, welche die Vereinigungsgeschwindigkeit von roten Blutkörperchen und Opsoninen bestimmen, unter der Annahme, dass 1. ein Adsorptionsprozess stattfindet, und 2. eine gegenseitige Fällung, von entgegengesetzter elektrischer Ladung abhängend, eintritt.

Nr.	Natur des wirksamen Bestandteils im roten Blutkörperchen	Natur des Opsonins	Vereinigungsgeschwindigkeit, abhängig von		Art der Reinigungsgeschwindigkeit	
			a.	b.	a.	b.
1.	Kolloid	Kristalloid	(Diffusion)	Kondensation	(monomolekular)	?
2.	„	Kolloid	Beimischung	Attraktion	?	?

Der dritte Fall (3. in Tabelle 3) ist ähnlich dem ersten und erfordert keine weitere Betrachtung.

Ausser der chemischen Veränderung bleibt noch die Möglichkeit, dass die Interaktion der roten Blutzelle mit Opsonin ein Adsorptionsprozess sei. Ein solcher kann nur eintreten, wenn der wirksame Bestandteil der roten Blutzelle ein Kolloid und das Opsonin ein Kristalloid ist (1. in Tabelle 3). Die Adsorptionsgeschwindigkeit würde sich aus zwei Faktoren zusammensetzen: 1. der Diffusionsgeschwindigkeit des Kristalloids gegen die Oberfläche des Kolloids; 2. der Kondensationsgeschwindigkeit des Kristalloids auf der Oberfläche des Kolloids. Die Adsorptionsgeschwindigkeit würde von der Diffusionsgeschwindigkeit abhängen, wenn die Kondensation relativ rasch ist, und würde dann eine logarithmische Funktion der Opsoninkonzentration sein; wenn dagegen aber die Diffusion die schnellere wäre, so würde die Adsorptionsgeschwindigkeit beherrscht werden von der Geschwindigkeit, mit welchem die Kondensation statthat. Die erste Alternative erweist sich nach den in Tabelle 2 gegebenen Daten als unhaltbar.

Der einzige noch übrig bleibende Modus, nach welchem eine Vereinigung der roten Blutzelle und Opsonin stattfinden kann, ist die, wobei der wirksame Bestandteil der roten Blutzelle und Opsonin Kolloide sind (2. in Tabelle 3), welche entgegengesetzte Ladung haben, und wodurch das letztere auf der erstern niedergeschlagen wird. In diesem Falle würde die Fällungsgeschwindigkeit bestimmt werden durch: 1. die Geschwindigkeit, nach welchem die kolloidalen Opsoninteilchen nach der roten Blutzelle angezogen werden, und 2. die Geschwindigkeit, nach welchem frische Opsoninteilchen in die Nähe der roten Blutzellen gehen und diejenigen ersetzen, welche sich an der roten Blutzelle festgesetzt haben; der letztere Vorgang ist vergleichbar mit der Diffusion; er ist aber molar

anstatt molekular. Keine dieser Geschwindigkeiten ist meines Wissens experimentell untersucht.

In der obigen Streitfrage wurde die Annahme gemacht, dass die an der Interaktion der roten Blutzelle mit Opsonin beteiligten Substanzen der Zahl nach zwei sind; kommen mehr als zwei Substanzen in Betracht, so wird das Problem komplizierter. Dann ist die Möglichkeit des Eintritts von Tri- oder Polymolekularreaktionen vorhanden. Bei der Bestimmung der Natur des wirksamen Bestandteils oder der Bestandteile der roten Blutzelle und Opsonin ist es jedoch nicht vorteilhaft, das Problem weiter zu verfolgen.

Der einzige Schluss, den die in Tabelle 3 aufgeführten Bestimmungen der Reaktionsgeschwindigkeit sicher zu stellen scheinen, ist der, dass alle die monomolekulare Geschwindigkeit umfassenden Reaktionen ausgeschlossen sind, eine Entscheidung aber unter den übrigen noch in Betracht kommenden Reaktionen zur Zeit unmöglich ist.

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On Opsonins in Relation to Red Blood-cells.

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(From the Lister Institute of Preventive Medicine.)

In a previous communication* it was pointed out that opsonins, whose existence in relation to bacteria is generally recognised, are also produced by injecting foreign red blood-cells into the peritoneal cavity. The latter variety of opsonin, which is thermostable, not being destroyed by exposure to 58° C. for 30 minutes, is taken up by red blood-cells of the kind used for injection. Such red blood-cells, after being thus "sensibilised" by opsonin, are ingested by leucocytes.

Since it appeared obvious that the occurrence of phagocytosis might, under suitable conditions of experiment, be made use of in order to discover if a serum were capable of sensibilising red blood-cells, that is, if it contained opsonin corresponding to the red blood-cells employed, it was determined to ascertain if, proceeding along the line of investigation thus suggested, a method for the estimation of red blood-cell opsonins could be devised. It was further decided to study the nature of the action of such opsonins upon red blood-cells.

Estimation of Opsonin.

In attempting to make use of phagocytosis for detecting the presence of opsonin in serum a serious difficulty is at the outset encountered owing to

* J. O. W. Barratt, "The Phagocytosis of Red Blood-cells," 'Roy. Soc. Proc.,' B, 1905, vol. 76, p. 524.

the occurrence of spontaneous phagocytosis. When the red blood-cells of the rabbit, for example, are injected into the guinea-pig, opsonin for such red blood-cells develops in the latter's serum, which (after destruction of complement (alexin) by heating for 20 to 30 minutes at 58° C.*) may now be used to sensibilise the red blood-cells of the rabbit. If the sensibilised red blood-cells are added to the leucocytes of the guninea-pig, contained in the (heated) normal serum of the guinea-pig, extremely active phagocytosis of the red blood-cells at once takes place. If, however, sensibilisation is omitted, phagocytosis is still observed, though in considerably diminished degree. Attempts to overcome the disturbing effect of this residual or "spontaneous" phagocytosis have not as yet met with a sufficient degree of success to enable estimations of opsonin to be carried out in such cases.

There is, however, another class of cases in which spontaneous phagocytosis does not occur. Leucocytes contained in the serum natural to them do not ingest the red blood-cells with which they are normally associated.† If, for instance, the red blood-cells of the guinea-pig are injected into the abdominal cavity of the rabbit and the serum of the latter is employed for sensibilisation, the leucocytes of the guinea-pig, contained in (inactivated) guinea-pig serum are now seen to ingest such sensibilised red cells, though if sensibilisation is omitted no phagocytosis whatever occurs.

A serum of this kind was prepared from a rabbit, which will henceforth be referred to as Rabbit A. The serum was used for the series of experiments recorded in the tables.

Rabbit A.—Weight, 2300 grammes. Intraperitoneal injections were made as follows:—

On the 1st day the red blood-cells of 7·5 c.c. of guinea-pig's blood.

On the 11th day the red blood-cells of 4·5 c.c. of guinea-pig's blood.

On the 21st day the red blood-cells of 4·5 c.c. of guinea-pig's blood.

On the 118th day the rabbit was bled to death. The serum obtained was inactivated by heating to 58° C. for 30 minutes.

An attempt was first made to ascertain if opsonin could be removed from the serum of Rabbit A by the washed red blood-cells of the guinea-pig, added in varying amounts and acting upon the serum (diluted and undiluted) for periods of time ranging from five minutes to five hours (Table I). During this time the mixture of serum and red cells was continually shaken, either

* Otherwise hæmolysis of the red blood-cells occurs.

† All the experiments with sensibilised red blood-cells, given in the tables, were accompanied with control experiments on normal red cells. In these, as in numerous other control experiments not referred to here, the results as regards phagocytosis were invariably negative.

by hand or in a motor-driven shaker. The mixture was then centrifugalised and the supernatant liquid pipetted off (great care being taken to ensure the complete removal of the red blood-cells).

The washed red blood-cells of 0·005 c.c. of guinea-pig's blood were next added to 1 c.c. of the deopsinated serum, and the whole agitated for two hours at 37° C. The sensibilised red cells, after being washed in 0·85 per cent. sodium chloride solution, were added in excess to the leucocytes of the guinea-pig in inactivated guinea-pig serum and kept on a warm stage (37° C.) for two hours. The leucocytes were then examined under the microscope and the percentage containing red blood-cells noted, 2000 to 4000 leucocytes being enumerated. The percentages thus obtained are only approximately correct,* as will be readily understood by reference to Table III, but by making a number of tests under conditions as nearly as possible identical in respect of every detail, fairly concordant results are obtained.

The results, given in Table I, showed that it was possible to remove opsonin from the serum employed, provided that a sufficient number of red cells were used. After the red blood-cells of one or more parts of guinea-pig's blood had acted upon two parts of the serum of Rabbit A, the serum had completely lost its power of sensibilising red cells; in other words, it no longer contained opsonin in recognisable amount. If the red blood-cells of one part of guinea-pig's blood were shaken with four parts of serum, sufficient opsonin was usually left in the serum to sensibilise red cells; this was especially the case when the period for deopsination was short. In Table I no indication of the degree to which opsonin is removed is afforded beyond the fact that a period of five minutes is too short to effect complete removal at 37° C.

The enquiry now presents itself, how much serum is required to sensibilise the red blood-cells of a given volume of guinea-pig's blood? To investigate this point the series of observations recorded in Table II was undertaken.

* The degree of phagocytosis obtained, apart from the degree to which sensibilisation of the red cells has been carried, depends chiefly upon the uniformity and the closeness of distribution of leucocytes and red cells. To secure the maximum degree of phagocytosis the cells must be evenly distributed and the leucocytes must not be separated by more than a few cell breadths from the erythrocytes, for leucocytes do not appear to recognise the presence of the latter until their processes come in contact with them. Difficulty was occasionally experienced owing to the occurrence of some degree of agglutination of the red cells after treatment with the rabbit's serum; separation could usually be effected by shaking, provided the proportion of serum employed was not too great (*i.e.*, $\frac{B}{S}$ not above $\frac{1}{20}$, compare Table III). The condition of the leucocytes also affects the degree to which the red cells are taken up. If a sufficiently extended series of tests are made the condition of the leucocytes tends to become the same in each experiment.

Table I.—Deopsination of Serum of Rabbit A, by means of the Red Blood-cells of the Guinea-pig. Temperature of Experiment, 37° to 38° C.

No. of expt.	Dilution of serum.	$\frac{B^*}{S}$.	Phagocytosis.† [The duration of the deopsinating action of the red blood-cells on the serum is given in brackets.]									
			[5 min.].	[10 min.].	[20 min.].	[40 min.].	[1 hr.].	[1½ hr.].	[2 hr.].	[3 hr.].	[4 hr.].	[5 hr.].
1	1 in 8	$\frac{8}{1}$							—			
2	Undiluted	$\frac{4}{1}$							—			
3	1 in 8	$\frac{2}{1}$							—			
4	Undiluted	$\frac{1}{1}$							—			
5	,,	$\frac{1}{1\cdot6}$				—	—		—			—
6	1 in 8	$\frac{1}{2}$							—			
7	1 in 2	,,			—		—				—	—
8	1 in 8	$\frac{1}{4}$							—			
9	Undiluted	,,							—			
10	,,	,,	+ + +		+ +			—		—		
11	1 in 8	,,	+ + +		+ + +			—		—		
12	Undiluted	,,	+ + +		+ + +			+ + +		+		
13	1 in 8	,,	+ + +		+ +			+ +		+		
14	Undiluted	,,	+ + +		—			—		—		
15	1 in 8	,,	+ + +		+ + +			+ + +		+ + +		
16	Undiluted	,,	+ +		+ +			+		+		
17	1 in 8	,,	+ + +		—			+ + +		+ + +		
18	Undiluted	,,			—			+ +		+ +		
19	1 in 8	,,			—			—			—	
20	,,	,,	+ + +	+ + +	+ + +	+ + +						
21	,,	,,			+ +	+ +						

* The washed red blood-cells of guinea-pig's blood (B) were added to the serum of rabbit A (S) in the proportions given. Thus, $\frac{B}{S} = \frac{8}{1}$ means that eight parts of guinea-pig's blood and one part of the serum of the rabbit were taken.

† In order to test phagocytosis, the red blood-cells of 0·005 c.c. of guinea-pig's blood were sensibilised with 1 c.c. of the deopsinated serum, diluted or undiluted, and then added to leucocytes obtained from the guinea-pig. The sign — means that no phagocytosis occurred; + that 0·2 per cent. to 0·7 per cent. of the leucocytes contained red blood-cells; + + that 1 per cent. to 1·5 per cent. contained red cells; + + + that the maximum degree of phagocytosis was obtained (2 per cent. to 5 per cent., in one instance 10 per cent., in another 20 per cent.).

In these experiments the red blood-cells of gradually diminishing amounts of guinea pig's blood were added to a given volume of the serum of Rabbit A, and the whole shaken for two hours at 37° C., the red cells being then washed and tested as to their power of exciting phagocytosis. It will be seen that so long as the ratio of the former to the latter exceeds 3 : 1 the red blood-cells are quite insufficiently sensibilised and fail to excite phagocytosis when added to the leucocytes of the guinea-pig. When the proportion is 1 : 1, phagocytosis becomes marked,* and no further increase in the

* The highest degree of phagocytosis obtainable was found to be that in which 2 to 6 per cent. of the leucocytes had taken up red cells at the end of 2 hours at 37° C. (after 2 hours little or no increase occurred). This range represents the variation to be expected under the most favourable circumstances. Percentages of 20, 12 and 10 were occasionally obtained, but were quite exceptional.

amount of serum employed causes any recognisable augmentation of the degree of phagocytosis obtainable. From this it may be concluded that, as far as phagocytosis is concerned, this ratio represents complete sensibilisation. This does not, however, represent the maximum amount of opsonin which can be taken up, for it appears, from Table I, that the red blood-cells of one part of guinea-pig's blood in some of the experiments removed opsonin to such an extent from four parts of serum that the serum was no longer capable of sensibilising red blood-cells, though in other experiments sufficient opsonin for this purpose was still left. An explanation of the difference in the amount of opsonin taken up in the two cases is probably to be found in the circumstances that phagocytosis is essentially a surface phenomenon depending upon the degree to which the outer part of the red blood-cell is sensibilised, and no advantage arises from the opsonin acting upon the cell in its whole thickness.

A striking fact becomes evident from Table II, namely that dilution of the serum does not arrest the process of sensibilisation of red blood-cells. When the red cells of one part of guinea-pig blood are mixed with one part (or more) of serum, sensibilisation occurs and phagocytosis results equally whether the serum is undiluted or is mixed with as much as 500 parts of 0·85-per-cent. sodium chloride solution.

The ratio noted above $\left(\frac{B}{S} = \frac{1}{1}\right)$ as representing the minimum relative amount of serum necessary for the production of marked phagocytosis enables an idea to be formed of the delicacy of the test of deopsination employed in Table I. If the red blood-cells of 0·005 c.c. of the blood of the guinea-pig are mixed with 1 c.c. of deopsinated serum, and are found to become so far sensibilised that marked phagocytosis is obtained, it follows that the serum contains, after deopsination, at least 0·5 per cent. of the amount of opsonin originally present. If, however, phagocytosis is found, on repeated testing, to be always feeble or to be absent, it follows that less than this percentage is present. By adding the red blood-cells of varying amounts of guinea-pig's blood to 1 c.c. of the serum, and noting the point at which sensibilisation becomes incomplete and phagocytosis feeble or absent—and, as is illustrated in Table II, this point is indicated with a fair amount of clearness if a sufficient number of experiments are made, the same technique being closely followed in each—it appeared to be feasible to ascertain approximately the degree to which opsonin had been removed from the serum in question.

Influenced by this line of thought, the series of determinations in Table III, which forms a continuation of Experiments 8 to 21 in Table I,

Table II.—Experiments to Determine the Degree to which Red Blood-cells must be
Rabbit A, and the Red Blood-cells

No. of experiment.	Duration of action of red blood-cells on serum.	At temperature of—	Phagocytosis* (the dilution						
			B* S = $\frac{32}{1}$.	$\frac{16}{1}$.	$\frac{8}{1}$.	$\frac{4}{1}$.	$\frac{3}{1}$.	$\frac{2}{1}$.	$\frac{1\cdot5}{1}$.
1	2 hours.	37° C.							— (1 in 256)
2	"	"							
3	"	"							
4	"	"							
5	"	"							+ (1 in 512)
6	"	"							
6	"	"							
6	"	"							
6	"	"							
7	"	"	— (1 in 14)	— (1 in 7)	— (1 in 7)		+ + (1 in 7)		
8	"	"			— (1 in 10)	— (1 in 10)		+ (1 in 7)	
9	"	"				— (1 in 10)		— (1 in 10)	
10	"	"				— (1 in 100)		+ + (1 in 100)	
11	"	"							

* Phagocytosis was tested by sensibilising the washed red blood-cells of guinea-pig's blood (B) with the serum the rabbit's serum were taken. After sensibilisation the red cells were washed in 85-per-cent. sodium 0·2 to 0·6 per cent. of the leucocytes ingested red blood-cells; + + that 0·8 to 1·3 per cent. contained instance 9 per cent., in another 10 per cent.).

was carried out. Portions of the serum of Rabbit A, diluted with an equal volume of 0·85-per-cent. sodium chloride solution, were mixed with the washed red cells of guinea-pig blood in the proportion of 4 : 1, for periods of time varying from 5 minutes to 3 hours and 20 minutes, and subsequently tested for the presence of opsonin, as in previous experiments (A to D, Table III). A second (E to H), and third (I to M) series of the same character were also carried out, the serum being diluted to 1 in 8 and 1 in 7 respectively. The tests of phagocytosis were made in groups, sometimes with diminishing amounts of the same serum (arranged horizontally in Table III), as in Experiments 1 to 18, sometimes with serum deopsinated for different periods of time (these experiments are marked with an asterisk

sensibilised in order to excite Phagocytosis. The Serum employed was that of were those of the Guinea-pig.

of the serum is given in brackets).								
$\frac{1}{1}$.	$\frac{1}{1 \cdot 5}$.	$\frac{1}{2}$.	$\frac{1}{3}$.	$\frac{1}{4}$.	$\frac{1}{6}$.	$\frac{1}{8}$.	$\frac{1}{12}$.	$\frac{1}{24}$.
	+ + + (1 in 128) + (1 in 512)		+ + + (1 in 64) + (1 in 256)		+ + + (1 in 32) + (1 in 128)		+ + + (1 in 16) + (1 in 64) + + + (1 in 16)	+ + + (1 in 8) + + (1 in 32) + (1 in 16)
	+ + (1 in 128) + + + (1 in 256)		+ + + (1 in 64) + + + (1 in 128)		+ + + (1 in 32) + + + (1 in 64)			
+ + + (1 in 8) + + + (1 in 16) + + + (1 in 32) + + + (1 in 512)								
+ + + (1 in 5) + (1 in 10) + + + (1 in 100)		+ (1 in 10) + + + (1 in 100)		+ (1 in 10) + + (1 in 100)		+ (1 in 10) + + + (1 in 100) + + + (1 in 100)		

of Rabbit A in the proportions given. Thus, $\frac{B}{S} = \frac{32}{1}$ means that 32 parts of guinea-pig's blood and 1 part of chloride solution and added to the leucocytes. The sign — means that no phagocytosis was observed ; + that red cells ; and + + + that the maximum degree of phagocytosis was reached (2 to 6 per cent., in one

and are arranged vertically in Table III) as in Experiments 19 to 30. In spite of the variations exhibited by individual experiments fairly definite limiting values are obtainable for most of the sera. Thus in the experiments with Serum A a sharp diminution of sensibilising power is seen when the ratio B/S is less than 1/25, whence it is concluded that 25 parts of serum after deopsination contain only as much opsonin as one part originally did, that is to say, the opsonin has been reduced to 4 per cent. of the amount present at the beginning of the experiment; with B and C a distinct but less marked change in the sensibilising power of the sera is seen, indicating reduction of opsonin to 2 per cent. and 1 per cent. respectively of the amount originally present; the opsonin in D is obviously reduced to less than

Table III.—Exhibiting the Degree of Deopsination of the Serum of Rabbit A, effected by the Red Blood-cells of the Guinea-pig acting for varying Periods of Time. Deopsination was effected by adding the washed Red Blood-cells of one part of Guinea-pig Blood to four parts of the Rabbit's Serum.

No. of experiment.	Serum.	Dilution of serum.	Time allowed for deopsination.	Temperature at which deopsination was effected.	Phagocytosis of red blood-cells of the guinea-pig, sensibilised by the deopsinated serum of Rabbit A. In carrying out these tests, the washed red blood-cells of one part of guinea-pig's blood were added to the deopsinated serum in the following amounts:—																							Approximate amount of opsonin still remaining, the amount originally present being taken at 100.
					$\frac{B}{S} = \frac{1}{500}$	$\frac{1}{250}$.	$\frac{1}{100}$.			$\frac{1}{60}$.		$\frac{1}{50}$.			$\frac{1}{32}$.	$\frac{1}{25}$.			$\frac{1}{16}$.	$\frac{1}{12}$.	$\frac{1}{8}$.	$\frac{1}{6}$.	$\frac{1}{4}$.	$\frac{1}{3}$.	$\frac{1}{2}$.	$\frac{1}{1}$.	
			h. m.		p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	per cent.
1	A	1 in 2	0 5	37° C.	4*			2*	20*						7				0		0		0		0	0		4
2	,, B	,,	,,	,,								12				10				0·3		0·2						
	,,	,,	0 20	,,	0*	0*	1*	3*	1*																			2
3	C	,,	1 20	,,	0*	1*	1·5*	2*	0*			2				0·5				0·5		0						
	,,	,,		,,				2		0			0															1
4	D	,,	3 20	,,	0*	1*	0·4*	0·5*	0*	0					0				0		0		0		0	0		
5	,,	,,	,,	,,												0·5												< 1
6																												
7	E	1 in 8	0 5	,,						10	2*				6	10*	2*			6		10		0				15
8	,,	,,	,,	,,																								
9	F	,,	0 20	,,						1·5	2*			0*	1	5*	1·5*	2*										4
10	,,	,,	,,	,,																		0·5		0				
	G	,,	1 20	,,							4*			0*		0*	1*	1*										1·5
11	,,	,,	,,	,,																0·7		0						
	H	,,	3 20	,,							2*			0*		0*	0·7*	0*										< 1·5
12	I	1 in 7	0 5	,,						5		10*	0*		8													12
13	,,	,,	,,	,,																								
14	J	,,	0 10	,,						6		6*	0*		1						4		0·7		0	0		> 2
15	,,	,,	,,	,,																								
16	K	,,	0 20	,,						2		5*	+*		3						1·4		0		0	0		> 3
17	L	,,	0 40	,,								4*	+*		0	0			0		0		0		0	0		2
18	M	,,	4 0	34°·5 C.								0*	0*		0	0			0		0		0		0	0		
		No of experiment.....			19	20	21	22	23		24	25	26	27		28	29	30										

The low percentages have not the same value as the high percentages, since any imperfection of technique, in particular such as would lead to lowering of vitality of the leucocytes, or would result in defective admixture of the cells, causes diminution of the percentage noted. Hence when experiments are repeated with the same serum, the higher percentages are the more accurate. The low percentages often obtained when the amount of serum employed is relatively high $\left(\frac{B}{S} = \frac{1}{100} \text{ to } \frac{1}{500}\right)$ are due to the occurrence of agglutination, which in such cases causes difficulty, especially if the red cells have been too strongly centrifugalised.

1 per cent. of its original value, but beyond this its titre cannot be further defined from the experiments made; and similarly with the remaining sera.

The experiments in Table III show that a very rapid removal of opsonin takes place during the first five minutes, and that subsequently the removal of opsonin becomes considerably slower, some of the opsonin originally present still remaining in Sera D and H even at the end of several hours. As would be expected, the removal rate is seen to be more rapid with the more concentrated serum (A to D) than in the more dilute serum (E to H, and I to M).

The experiments in Table III show that, when spontaneous phagocytosis is avoided, the estimation of red blood-cell opsonin may be carried out. Sera from different sources may be compared as to their opsonic content by determining the minimum amount of serum required to sensibilise a given bulk of red cells so as to produce the maximum degree of phagocytosis An alternative method is to determine the minimal bulk of red blood-cells required to deopsinate unit of volume of the sera.

The Interaction of Red Blood-cell and Opsonin.

It is not possible from the data above given to arrive at a definite conclusion as to the nature of the process occurring when red blood-cells are sensibilised by opsonin.

It has been already pointed out that even considerable dilution of the serum of Rabbit A did not abolish sensibilisation so long as the ratio $\frac{B}{S} = 1$ was preserved. This circumstance is readily explicable on the assumption that a chemical reaction takes place, but it is also equally well explicable on the assumption that a physical process of adsorption or selective solubility occurs. The relation between concentration and adsorption has been determined by Ostwald* to be

$$C_1 = aC_2^n,$$

where C_1 is the concentration of the adsorbed substance, C_2 that of the substance in solution, and a and n are constants. By suitably choosing the values of a and n, the value of C_1 could be left practically unchanged when C_2 was reduced to 1/500 of its initial value.† So far as the behaviour of opsonins in respect of dilution has up to the present been investigated,

* 'Lehrbuch d. allg. Chemie,' 1891, vol. 1, 2te Auflage, p. 1096.

† For example, if $a = 1{,}000{,}000$ and $n = 1$, in which case (since $\frac{C_1}{C_2} = \frac{1000000}{1}$) all but 1×10^{-6} of the opsonin originally present in the liquid would pass to the red blood-cells. If n were less than 1, the proportion taken up by the red cells would be still greater. Unfortunately the estimation of very small percentages of opsonin requires much larger amounts of serum than can be obtained from rabbits.

Table IV.—The Character of the Interaction of Erythrocytal Opsonins with red Blood-cells. Temperature of Experiment 37° C. (Compare Table III.)

Experiments.	Dilution of serum.	Value of—				Relative value of—				Period.	Values of k, calculated from the formulæ—			
		t_0.	t_1.	t_2.	t_3.	C_0.	C_1.	C_2.	C_3.		$\frac{1}{t''-t'} \log \frac{C'}{C''}$.	$\frac{1}{t''-t'}\left(\frac{1}{C''} - \frac{1}{C'}\right)$.		$\frac{1}{t''-t'}\left(\frac{1}{[C'']^2} - \frac{1}{[C']^2}\right)$.
A to D, Table III	1 in 2	0′	5′	20′	80′	100	4	2	1	t_0 to t_1	0·0219	0·0025	mean 0·0016	0·0017
										t_0 to t_2	0·0085	0·0025		0·0125
										t_0 to t_3	0·0025	0·0012		0·0063
										t_1 to t_2	0·0020	0·0017		0·0125
										t_1 to t_3	0·0012	0·0012		0·0062
										t_2 to t_3	0·0005	0·0008		0·0125
E to H, Table III	1 in 8	0′	5′	20′	80′	100	15	4	1·5	t_0 to t_1	0·0165	0·0011	mean 0·0010	0·0005
										t_0 to t_2	0·0699	0·0012		0·0016
										t_0 to t_3	0·0022	0·0008		0·0028
										t_0 to t_2	0·0028	0·0012		0·0020
										t_1 to t_3	0·0013	0·0008		0·0030
										t_2 to t_3	0·0007	0·0007		0·0032
I to M, TableIII	1 in 7	0′	5′	40′	—	100	12	2	—	t_0 to t_1	0·0186	0·0025	mean 0·0016	0·0069
										t_0 to t_2	0·0425	0·0012		0·0033
										t_1 to t_2	0·0020	0·0012		0·0035

the results obtained do not permit a conception of the nature of the interaction of red cell and opsonin to be formed.

In a stoichiometric reaction which proceeds slowly, not only is it possible to establish the molecular character of the reaction by means of determinations of the reaction rate, but the degree of the reaction may also be ascertained. The general formulæ for stoichiometrical reactions are

$$t''-t' = k \log C'/C''$$

for monomolecular reactions, and

$$t''-t' = k\,[1/(C'')^n - 1/(C')^n]$$

for multimolecular reactions, where C' is the concentration at time t', C'' that at time t'', n is unity for bimolecular reactions, and k is a constant. The truth of these formulæ has been established for homogeneous systems. Whether they are applicable to heterogeneous systems as in the interaction of toxin and antitoxin has been called in question. In the case of the heterogeneous system formed by red blood-cell + opsonin, the data above given enable the applicability of the formulæ in question to be tested empirically. In Table IV, from the data furnished by Table III which are repeated, the values of k are calculated: (1) as for a monomolecular reaction; (2) for $n = 1$; and (3) for $n = 2$.* The values of k for the first and third formulæ exhibit wide variations; the values for $n = 1$ show a fair correspondence, the average of all the experiments being 0·0014, while the highest and lowest values are 0·0008 and 0·0025 respectively. Thus the striking result is obtained that in the heterogeneous system under consideration a relation is exhibited similar to that observed in a bimolecular stoichiometric reaction occurring in a homogeneous system.

Summary.

It has been shown that—

1. By employing phagocytosis as a test of the presence of red blood-cell opsonin, and avoiding spontaneous phagocytosis by suitable conditions of experiment, quantitative determinations of the opsonic content of serum may be made.

2. In the experiments made, the interaction of opsonin and red blood-cell proceeded at a rate corresponding to that exhibited in a bimolecular stoichiometric reaction.

In conclusion, I must express my indebtedness to the assistance I have received from Dr. Allan Macfadyen, in whose laboratory this research was conducted, and from Mr. J. A. Craw and Professor Donnan.

* The concentrations of red blood-cell and of opsonin are regarded as equivalent, since the opsonin is present in the minimum proportion necessary to lead to full sensibilisation of the red blood-cells.

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OBSERVATIONS ON THE BACTERIOLOGY OF PARATYPHOID FEVER AND ON THE REACTIONS OF TYPHOID AND PARATYPHOID SERA.

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During the last eight years the existence has been established of a sub-acute bacterial septicaemia clinically resembling typhoid fever, but associated with the presence in the body of organisms which are closely allied to Gaertner's food-poisoning bacillus and amply distinct from the *B. typhosus.* The cultivation of a "paracolon" bacillus from the blood of a patient "with all the clinical features of typhoid fever," by N. B. Gwyn in 1898, represents the first clear diagnosis of a case of this paratyphoid fever, though the same organism had been already isolated by Achard and Bensaude (1896) from the urine of a case of apparent typhoid and from a purulent arthritis following a similar illness, and Widal and Nobécourt (1897) had found it in a thyroid

abscess arising in a patient apart from any symptoms of general infection. The observations of Cushing (1900), followed by those of Buxton (1902), Libman (1902) and others (see Johnston 1902 and Pratt 1903), established the position of paratyphoid fever as a definite, and not very uncommon, disease in America, while Schottmüller (1900, 1901), by the systematic application of the method of blood-culture in cases of "typhoid," showed that a similar condition obtained in Germany. Sporadic cases, with occasional epidemics (de Feyfer and Kayser 1902, Fischer 1903, Schottelius 1905) have since been described from many parts of the world, and the disease is probably of general distribution. In this country, however, only four cases seem to have been recorded, if we exclude such indefinite instances as those mentioned by Parsons[1] and Cautley[2]. Craig and White (1902) isolated from the spleen *post mortem* of "a case of continued fever resembling enteric, and due to the *B. enteritidis*," an organism which was probably a paratyphoid rather than a Gaertner; Hume's (1902) "bacillus L," obtained from the stools and urine of a "typhoid," seems to have belonged to the paratyphoid group; and Savage (1905) has recently described two cases of the disease, in one of which *B. typhosus* was isolated from the urine. As will presently be pointed out, paratyphoid bacilli cannot be definitely differentiated from other closely related forms without the use of agglutination tests with sera of known origin. It is unfortunate that these tests have not been applied in any of these four cases. It may therefore be worth while to put on record here three instances of paratyphoid fever which have been met with during the past year, together with two further cases in which the existence of a similar condition was highly probable though not fully proven.

I. *Methods of investigation employed.*

In dealing with organisms, such as the present, where distinctions of some narrowness have to be drawn and where details which at present appear immaterial may hereafter become of importance, it is desirable to give some account of the methods and standards employed.

(1) *Media.* For plates, the lactose-bile-salt neutral-red agar of MacConkey (1905, p. 334) has been exclusively used, and has been found altogether satisfactory. For identification, besides the customary media, much use has been made of peptone water containing neutral red or

[1] *British Medical Journal*, 1905, vol. I. p. 305.

[2] *British Journal of Children's Diseases*, 1905, vol. II. p. 241.

litmus with 0·5 or 1 per cent. of various carbohydrates, alcohols, and glucosides, and filled into Durham's fermentation tubes.

(2) *Isolation.* Direct plating of stools may be successful, but, as always in working with such material, *B. coli* is much in the way. A differential medium which will encourage the growth of paratyphoid more than that of *B. coli* is of assistance. Such a medium is found in the dulcite-bile-salt peptone water of MacConkey. It is characteristic of the whole group of paratyphoid and food poisoning organisms that they ferment dulcite freely[1]. This fermentation is accompanied by proliferation, so that in dulcite broth dulcite-fermenters tend to overgrow non-dulcite-fermenters, just as in lactose broth the *B. coli* still further outnumber the non-lactose-fermenters. In the following experiment, approximately equal quantities of paratyphoid B[2] (fermenting dulcite but not lactose) and "*coli* 1" (fermenting lactose and dulcite but not cane-sugar: *B. coli communis*) or "*coli* 4" (fermenting lactose but not dulcite or cane-sugar: *B. acidi lactici*) were inoculated into plain broth, lactose peptone water, and dulcite peptone water; after 20 hours' incubation at 37° C. plates were made and the relative numbers of each organism now present determined.

	P.c. found on plates	
	Coli	Paratyphoid
Paratyphoid + *coli* 1 incubated in plain broth	19	81
,, ,, ,, lactose	96	4
,, ,, ,, dulcite	18	82
Paratyphoid + *coli* 4 incubated in plain broth	95	5
,, ,, ,, lactose	99	<1
,, ,, ,, dulcite	1	99

The dulcite-fermenters present in faeces comprise about half the *B. coli* (A. C. Houston (1904, p. 544) and A. MacConkey (1905)), certain members of the food-poisoning group isolated by H. de R. Morgan (1905), and two or three other unnamed varieties of "*coli*-like" organisms: the last two are not abundant. They are therefore far less abundant than *e.g.* lactose- or dextrose-fermenters, and by means of this preliminary differential culture in dulcite media paratyphoid B was isolated from the stools in two cases in which direct plating had given altogether negative results.

[1] This important reaction seems to have been first noted for hog-cholera by Voges and Proskauer (*Zeitschrift für Hygiene*, 1898, vol. XLVIII. p. 20) and for paratyphoid by Conradi, Drigalski and Jürgens (1903).

[2] The strain used was "*Potts*" (see below). All three organisms were recently isolated from human faeces.

The highly pathogenic relations of these organisms suggests another convenient method of isolation, and after inoculation of guinea-pigs with infected faeces (and especially with cultures of faeces in dulcite broth) the organisms may often be obtained in abundance from the heart-blood. There is, however, evidence that similar organisms may be recovered from the blood of animals inoculated with material undoubtedly free from any such bacillus; thus A. MacConkey (1905, p. 343, *note*) obtained bacilli culturally resembling paratyphoid B[1] after the inoculation of dead typhoid bacilli. It is clear from this that the method cannot be used unless corroboration be obtained without the use of animals.

Circumstances have in the present cases precluded the use of direct cultivation of the patient's blood which seems to be at once the simplest and most accurate method of isolation.

(3) *Agglutination reactions* have in all cases been observed microscopically in the hanging drop. This was in the present instance necessary owing to the small quantities of serum often available; apart from this, however, it seemed desirable to make use of the more delicate method. Broth cultures of the organisms grown for about 20 hours were used. Such preparations as dead formalinised cultures might have afforded a more uniform test-standard, but a series of preliminary experiments, in which the agglutinabilities of such preparations and of fresh living cultures were compared, gave results distinctly unfavourable to the dead bacilli as regards delicacy as well as rapidity of reaction. It appeared too that the difference between the dead and living bacilli was not relatively the same with different sera (human), some sera showing no difference, while others had much less action on the dead than on the live organisms. The same serum shows much less (and absolutely quite little) variation when reacting with a number of live cultures grown under similar circumstances of time, temperature, and medium. The most desirable temperature for growth varies with the different organisms used, and must be determined by experiment; thus one typhoid ("Guy's") at 20° grows into long threads, while another ("Lincoln") is practically non-agglutinable if grown at 37°. In the same way some paratyphoid and food-poisoning organisms grown at 37° are very prone to spontaneous agglutination; this is not present in a growth of 5 or 6 hours at 42° or

[1] Probably belonging to the hog-cholera group which produces one form of pseudo-tuberculosis of guinea-pigs.

of 24 hours at 20°. In other cases it seems to be immaterial whether the culture has been at 20°, 37°, or 42°. The customary series of serum-dilutions in 0·9 per cent. NaCl at which observations were made was 1 in 20, 1 in 50, 1 in 100, 1 in 500, 1 in 1,000, 1 in 5,000, and 1 in 10,000: the wide spacing of the higher dilutions is justified by the unfortunate lack of sharpness in the end-point which is inherent in the reaction[1]. Observations were made after the mixtures had stood for half to one hour at room temperature; in all comparative tests the time-limit was the same throughout the series. Control preparations were made in all cases. The degree of reaction has been noted in five grades:—complete clumping = + + +; nearly complete but some free bacilli = + +; a fair number of small clumps with many free bacilli = +; a few clumps composed of perhaps not more than three or four organisms = tr.; same as control = 0. The agglutination limit of sera failing to react at 1 in 20 is generally described as 0. The refusal to recognise reactions other than those of a "complete" kind fails to take into account phenomena which control experiments show to be of a perfectly definite nature. Since the range of serum-dilution required to reduce a "complete" to a "trace" reaction varies a great deal with different combinations of bacilli and sera, this may lead to altogether erroneous results. The degree of reaction must be estimated by comparison with the appearances presented by the most complete reaction obtainable with the given organism and kind of serum. This differs a good deal in different combinations. Thus Gaertner and paratyphoid B show only loose, relatively open aggregations of bacilli with any dilution of their respective immune sera. These might be regarded as only partial reactions if judged by the conventional standard of the dense clumps formed by typhoid bacilli in typhoid serum. The highest dilution of a serum at which a reaction is obtained has throughout been taken as quantitatively representing the content of the serum in agglutinin.

(4) *Absorption tests.* The absorbing organisms have been agar cultures of (generally) two days' growth; the serum dilution 1 in 10 to 1 in 500, and the time two or three hours at 37°. Nothing is gained by longer contact except a rapid deterioration of the serum; as a matter of fact one hour seems to be ample. In some instances the agglutination limits of the serum were redetermined after absorption; with a combination,

[1] As illustrating the quantitative limitations of the phenomenon the observations of G. Dreyer and A. J. Jex-Blake (*British Medical Journal*, 1904, vol. II. p. 564) should be consulted.

however, of many absorbing organisms and many agglutinable bacilli, the test becomes very extensive, and in the majority of cases the diluted serum has been mixed with each organism (added if necessary in successive amounts) until it ceases to react with that bacillus or reacts with that bacillus alone: its reaction with the other bacilli is then observed qualitatively at a definite dilution. By thus saturating the serum by the addition of an excess of bacilli, difficulties are avoided in the measurement of comparable quantities of organisms differing in size, texture and fluidity, which are inherent in the method by which "equal" quantities of bacilli are added to equal quantities of serum and the relative disappearance of agglutinin estimated. For many of the problems this simpler method will not of course suffice; for the applied purposes of diagnosis it is very satisfactory.

If the agglutination limits are determined after absorption, some allowance is necessary for the inevitable and unknown dilution produced by the addition of water with the absorbing bacilli and for the deterioration of agglutinating power which is caused by incubation at 37° for several hours (cf. Park and Collins 1904, p. 500). The influence of this latter factor seems inconstant: it may be very marked.

II. *Description of organisms concerned.*

The following is a list of the strains of the various standard organisms which have been used[1]: the names by which they are referred to below are in italics.

A. Typhoid.

1. *Guy's:* the standard agglutinating strain of this laboratory.
2. *Lincoln:* recently (June 1905) isolated from the urine of a typhoid convalescent.
3. *A. E. Wright:* old laboratory strain.
4. *Delépine:* " " "

The cultural and agglutination (including agglutinogen) reactions of all four strains were typical. The fermentative reactions were: acid without apparent gas in dextrose, maltose, laevulose, galactose, sorbite, and mannite, but no change in lactose, cane-sugar, dulcite, raffinose, arabinose, erythrite, salicin, amygdalin or inulin. They differed somewhat in their action on litmus milk; all produced a definite acidity

[1] The cultural characters and agglutination reactions of most of these have recently been fully described by H. de R. Morgan (1905) and fuller details of the fermentative properties of some of them are given by A. MacConkey (1905).

in 24 hours; in 9 days *Lincoln* and *A. E. Wright,* and in 15 days *Delépine,* were markedly alkaline; *Guy's* did not produce definite alkali till after a month's incubation.

B. Paratyphoid and food-poisoning group.

(*a*) Paratyphoid A.

1. *Schottmüller A:* original strain (1901).
2. *Brion and Kayser* (1902).

(*β*) Paratyphoid B.

Schottmüller B: original strain (1901).

(*γ*) Hog-cholera.

Aertryck: isolated from an outbreak of food-poisoning: from Prof. van Ermengem[1].

(*δ*) Gaertner.

1. *Gaertner L.I.P.M.:* old laboratory strain.
2. *Gaertner original A:* original strain from Prof. Gaertner.

For the most part all the members of this group B show identical cultural reactions. They are actively-motile "coli-like" organisms; no liquefaction of gelatine; indol none or in small or moderate amount;

acid and gas in	*dextrose*	dextrin[2]
	laevulose	mannite
	maltose[3]	*dulcite*
	galactose	*sorbite*
	arabinose	

but no apparent change in

lactose[4]	*salicin*
cane-sugar	amygdalin
raffinose[5]	inulin
erythrite	

[1] See van Ermengem in Kolle and Wassermann's *Handbuch,* 1903, vol. II. p. 657.

[2] The absence of dextrin fermentation in the paratyphoid B isolated by H. G. Wells and L. O. Scott (1904) is probably accounted for by the variation of the so-called "dextrin."

[3] Drigalski (1903, p. 421) found that *Aertryck* produced no change in maltose after repeated trials.

[4] H. Kayser (1904 *b*) describes paratyphoid B (but not A) as producing gas in lactose broth as does W. Korte (1903). A. Brion and H. Kayser (1902) in the original description of their organism say that it produces a small amount of gas in lactose broth.

[5] A. MacConkey (1905, p. 350) states that paratyphoid A and B produce acid and gas in

By their action on litmus-milk (or litmus-whey) they fall into two distinct classes: (1) paratyphoid A producing permanent[1] acidity without clotting; (2) paratyphoid B, hog-cholera and Gaertner producing an initial slight acidity which is succeeded after a very variable interval (2 to 14 days) by definite and increasing alkalinity.

The production of "indol" is variable[2]. Inasmuch, however, as small amounts are very frequently found in 5-day old broth cultures of paratyphoid B and hog-cholera, and very rarely (if at all) in similar cultures of Gaertner, a positive reaction may be of some value in diagnosis. A negative result is of less import.

The groups paratyphoid B, hog-cholera and Gaertner are then practically indistinguishable from one another by cultural methods. The work of H. Bruns and H. Kayser (1903), H. Trautmann (1903), H. de R. Morgan (1905), V. Porcile (1905) and others shows, however, that by their agglutination reactions they may be readily separated. It is also clear that *for the satisfactory identification of any of these organisms it is necessary to examine their reactions with sera prepared with known cultures*[3]. By these tests Gaertner is separated from paratyphoid B and from hog-cholera almost as clearly as is paratyphoid A from any of the other three. In most instances there is also not much difficulty in dividing paratyphoid B from hog-cholera. Indications are, however, not wanting that the line of separation between these two is

raffinose: he has been good enough to tell me that acidity of the medium (containing 0·5 p.c. sodium taurocholate and tinted with neutral red) to a greater or less degree was always noted: gas formation was sometimes present, but more frequently absent; when present it was only in slight amount. This I have failed to confirm with Kahlbaum's raffinose in litmus peptone-water as far as two strains of A and four of B are concerned. H. Conradi, W. v. Drigalski and G. Jürgens (1902) describe *Schottmüller B* and a strain of paratyphoid B isolated by themselves as without action on raffinose, and Drigalski (1903) found that no change is produced in this medium by paratyphoid B or Gaertner.

[1] On two occasions, both *Schottmüller A* and *Brion and Kayser* have, after about two months, become strongly alkaline. Libman (1902) found the same with Schottmüller's "Müller" (*i.e.* A) strain. The difference may after all be only one of temporal degree: this does not however introduce any practical difficulty.

[2] Most observers, *e.g.* Schottmüller (1901), Kranepuhl (1905), Kayser (1904 *b*), Korte (1903), have stated that no indol is produced by any of these organisms. Among others Morgan (1905), Libman (1902), Buxton (1902), Cushing (1900) and Savage (1905), note the production of small quantities.

[3] It may be hardly necessary to point out that in such experiments it is not enough to show that an unknown organism reacts at some arbitrary dilution with a known serum; the ultimate limits of agglutination should be determined for each serum with both the unknown organism and the strain and organism homologous with the serum. The diagnosis depends on the relative, not the absolute, value of the figures so obtained.

not always very well-defined. Thus Bruns and Kayser (1903) note that *B. breslaviensis* (which Morgan (1905) has shown to belong to the hog-cholera (*Aertryck*) group) reacts with *Schottmüller B* serum to a dilution limit not much less than that for its homologous organism; and Trautmann's (1903) tables make it clear that paratyphoid B is much closer to hog-cholera than is Gaertner to either. Similar results are shown in the following table, which gives the agglutination limits of the three organisms with nine sera:

	Serum	*Schottmüller B*	*Aertryck*	*Gaertner L.I.P.M.*
I.	Paratyphoid B; human	20,000	20,000	<100
II.	,, ,,	1,000	500	50
III.	,, ,,	5,000	1,000	200
IV.	*Schottmüller B*; rabbit	5,000	1,000	200
V.	,, ,,	5,000	200	200
VI.	*Aertryck*; rabbit	100	5,000	<50
VII.	,, ,,	2,000	2,000	20
VIII.	*Gaertner original A*; rabbit	100	50	20,000
IX.	,, ,,	20	20	5,000

H. Bonhoff (1904) goes further and concludes (on inadequate grounds) that paratyphoid B cannot be distinguished even from Gaertner. H. Smidt (1905) has published agglutination reactions showing that hog-cholera, paratyphoid B, and *B. typhi-murium* are very closely allied and far removed from paratyphoid A: from his results the specific distinction of *B. typhi-murium* (which according to Morgan (1905) belongs to the Gaertner group) and paratyphoid B would seem doubtful[1].

On the whole, the distinction between hog-cholera and paratyphoid B, though slender, seems to be real. The morbific relations to man are different, for while hog-cholera (*Aertryck, breslaviensis*, Hatton (Durham 1898 *b, c*), Düsseldorf (Trautmann 1903)) gives rise to a sudden acute illness (food-poisoning), paratyphoid B causes a disease with no clear clinical distinctions from enteric fever[2], and to which the sequelae of an attack of food-poisoning do not bear any close resemblance. Such a difference must needs carry considerable weight unless we hold with Trautmann (1904) that food-poisoning is only a very acute infection and intoxication (organisms + toxins) with the same essential cause as paratyphoid fever (organisms only).

[1] As a point of technique, it may be pointed out that these differences in agglutination are best brought out by sera from animals which have received many inoculations (see below p. 50, and Fox, 1905 *b*).

[2] See however Schottmüller (1904) on the rôle of these organisms in the causation of acute gastro-enteritis.

The protective results of vaccination also indicate a clear distinction between paratyphoid B and *Aertryck*. A series of guinea-pigs of about 200 gms. were inoculated subcutaneously with 0·1 c.c. of a 20 hours' culture of paratyphoid B (strain *Schottmüller B*) killed by heating at 60° for 30 minutes. Nine days later they were tested by intraperitoneal injection of living cultures of *Potts* and *Aertryck*; the latter alone was rapidly fatal[1]:

Potts			*Aertryck*		
Control	0·01 c.c.	dead <20 hrs.	Control	0·5 c.c.	dead <20 hrs.
,,	0·001 ,,	dead 28 hrs.	,,	0·1 ,,	lived
Vaccinated	0·01 ,,	lived	Vaccinated	2·0 ,,	dead <17 hrs.
,,	0·01 ,,	dead 25 days	,,	1·0 ,,	,, ,,
,,	0·002 ,,	dead 36 days	,,	1·0 ,,	,, ,,
,,	0·001 ,,	lived	,,	0·5 ,,	,, ,,

As was pointed out by Durham (1898 *b*) some time since, the quantitative measurement of the degree of relationship of two or more organisms by their interactions with various sera is not free from fallacy unless the bacilli concerned are closely related by other characteristics. Such results as are shown in the following table are very frequently

	Agglutination limits with			
Serum	*Guy's*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M*
Schott. B; rabbit	1,000	10,000	200	50
,, ,,	2,000	5,000	200	200
Gaertner L.I.P.M.; rabbit	1,000	20	20	5,000
,, *orig. A*; rabbit	2,000	100	50	20,000

obtained and might be taken as showing that paratyphoid B is more nearly related to typhoid than to hog-cholera. If, as seems necessary, cultural (biological) characters are to form the basis of our classification, such is of course by no means the case. The *B. typhosus* belongs to an entirely different group though it has affinities which will find available agglutinin in many different "immune" sera, or, perhaps better, it is an organism of high agglutinability.

[1] From other experiments it appeared that the specificity of such protection was not high; thus vaccination with Gaertner produced a distinct resistance to paratyphoid B. Sacquépée and Chevrel (*Comptes Rendus de la Société de Biologie*, 1905, vol. LIX. p. 598) have recently shown that the mutual heterologous protective powers of typhoid and paratyphoid A and B vaccinations are almost as great as the homologous.

III. *Occurrence of paratyphoid fever.*

During a period of eight months (February to October) specimens of blood from 347 patients were received at the Institute to be examined for the Widal reaction. The majority came from London and the adjacent districts. They were presumably in all cases derived from persons suffering from an illness bearing at least some resemblance to typhoid fever. Of these, 176 (51 per cent.) gave the serum reactions of typhoid, 166 were negative, and in five instances the diagnosis of paratyphoid infection was suggested as the result of the examination. Three of these were shown to be cases of infection with paratyphoid B, and the organism was isolated from the stools; the other two were probably of the same nature, though definite proof was not obtained. It has been the universal experience that infections with paratyphoid B are far commoner than with paratyphoid A: thus Pratt (1903) in his analysis of 84 cases found only 12 instances of A infection, and Zupnik (1905) had 21 cases of B and only one A. An abstract of the recorded A cases is given by Kayser (1904 *a*), since when practically all published cases have been B infections.

We thus find that about 3 per cent. of the cases of "typhoid" are in reality due to an organism belonging to a different group—a proportion small enough but sufficient to show that paratyphoid fever occurs as something more than a bacteriological curiosity in this country. If the observed proportion is approximately representative, it follows that, of the 3,000 odd cases of typhoid notified each year in London, nearly 100 should be separated as not due to Eberth's bacillus. The proportion is probably, if anything, too low: Schottmüller (1901) in Hamburg found five paratyphoids in 68 "typhoids" and Zupnik (1905) 7 per cent. in 300. Dr H. G. Wells of Chicago tells me that systematic blood-cultures show that about 10 per cent. of the "typhoids" in that city are really paratyphoids, and Kolle (1905) estimates the proportion in Germany at about the same.

The case mortality of the 84 cases of paratyphoids analysed by J. H. Pratt (1903) was 3·5 per cent., and H. Kayser (1903) estimates it at only 1 to 2 per cent. This is in marked contrast with the 17 per cent. found for typhoid in London, and it follows that an unusual prevalence of paratyphoid might render the figures for the fatality of typhoid materially inaccurate. The accurate diagnosis of the condition

is therefore of more than pathological interest; the favourable prognosis and the frequent absence of intestinal ulceration (Lucksch 1903, Wells and Scott 1904), should show a distinct reaction in the increased comfort of all concerned with such cases from the more strictly medical point of view.

Clinically the five present cases were all rather mild, and perhaps in two cases rather indefinite, typhoid. They all ended in recovery. It is somewhat curious that four of the cases occurred in boys of $1\frac{10}{12}$[1], 4, 8 and 13 years: the fifth (the precise nature of which has not been fully determined) was in a woman aged 45. They all came from separate localities—Epsom, Woolwich, Balham, Lancaster and Southfields (the infection in the last case having been clearly contracted in Dublin), and came under notice in March, June, July, August and September[2].

A detailed account of the bacteriological findings in these cases will be appropriately preceded by some general considerations with regard to the serum reactions in typhoid and paratyphoid infections and their use in diagnosis.

IV. *The secondary agglutinations of typhoid sera.*

It is well-known that typhoid immune serum may agglutinate other organisms as well as the *B. typhosus*, and that sera prepared by infection with these other organisms may agglutinate typhoid as well as their homologous bacilli. Such sera may be said to contain, besides the homologous, specific or primary agglutinin, heterologous, secondary or so-called "group" agglutinins. Some idea of the frequency of these subsidiary reactions may be gained from the figures obtained in the present series of tests on human sera, no serum being tested at a lower dilution than 1 : 20.

[1] G. B. Allaria (1903) records a case in a child of two years; the organism is, however, described as fermenting lactose and a good deal of doubt is thus thrown on the diagnosis. Count and Kirby (1904) describe a fatal case in a child of $4\frac{1}{2}$ months; the organism obtained did not ferment mannite, laevulose or maltose, and there seems to be no reason for regarding the case as one of paratyphoid infection. Feyfer and Kayser's (1902) case, aged two years, was paratyphoid only by inference.

[2] During the six months, February to August, 44 per cent. of the sera gave typhoid reactions and four cases of paratyphoid occurred to 98 cases of typhoid; in the next two months only one case of paratyphoid was found to 78 typhoid and 62 per cent. of the sera became "positive" as the prevalence of enteric increased.

Organism[1]	No. of sera tested	Found to be typhoid	Secondary agglutination positive
Typhoid: *Guy's*	347	176	—
Paratyphoid A: *Schott. A*	347	176	19 = 10·8 p.c.
,, *Brion and Kayser*	157	101	58 57·4
Paratyphoid B: *Schott. B*	347	176	55 31·2
Hog-cholera: *Aertryck*	156	101	31 30·7
Gaertner: *L.I.P.M.*	194	125	64 51·2

The details of 86 consecutive typhoid sera which were tested with all six organisms, and in which the agglutination limits for typhoid were fully worked out, are given in the next table. Sera giving no reaction at 1 in 20 are recorded as negative.

Agglutination limits with						
Guy's	*Schott. A*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*	
20	—	—	—	—	—	21 cases
50	—	—	—	—	—	12 cases
	—	20	—	—	20	
	—	20	—	20	—	
	—	50	20	—	20	
100	—	20	—	—	—	
	—	20	—	—	20	
	—	20	—	—	50	
	—	20	—	—	50	
	—	20	20	—	20	
	—	100	20	—	20	
	—	20	20	20	20	
200	—	—	—	—	20	
	—	50	—	—	20	
	—	50	—	—	20	
	—	50	—	—	50	
	—	—	20	—	100	
	—	100	20	—	50	
500	—	—	—	—	—	
	—	50	—	—	—	
	—	20	—	—	20	
	—	50	—	—	50	
	—	50	50	—	200	
	—	200	50	20	200	
	20	50	100	20	20	

[1] The sera were at first tested only with typhoid, *Schottmüller A* and *Schottmüller B*: *Gaertner* and subsequently *Aertryck* were added, and *Brion* and *Kayser* used as well as *Schottmüller A* when the very low inherent agglutinability of this latter strain became known by tests with artificial homologous sera.

Agglutination limits with					
Guy's	*Schott. A*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*
1,000	—	200	—	—	200
	—	100	50	—	500
	—	200	100	—	500
	—	50	20	20	50
	—	50	50	20	50
	—	50	20	20	200
	—	50	20	20	200
	—	50	500	50	100
	20	50	100	20	100
	20	500	20	20	500
	20	500	50	50	500
2,000	—	—	—	—	—
	—	20	—	—	200
	—	100	50	20	20
	—	500	20	20	1,000
	—	1,000	50	50	100
	20	200	200	100	200
5,000	—	100	—	—	200
	—	500	2,000	500	—
	—	200	100	100	200
	—	100	100	50	200
	—	100	100	100	200
	20	100	50	50	200
	50	100	100	100	1,000
	50	2,000	200	200	500
10,000	—	—	100	200	1,000
	—	200	20	20	200
	—	200	200	200	500
	—	200	200	500	5,000
	50	200	200	200	500

Of this series, 51 (59 per cent.) showed secondary agglutinations while 35 (41 per cent.) reacted with typhoid only; 55 per cent. reacted with *Gaertner L.I.P.M.* or *Brion and Kayser*, 41 per cent. with *Schottmüller B*, 33 per cent. with *Aertryck*, and 12 per cent. with *Schottmüller A*. The titres of these coagglutinations and the combinations in which they occurred are summarized in the next tables.

	Number of cases which reached an agglutination limit of						
	20	50	100	200	500	1000 and over	Total
Schottmüller A	7	3	—	—	—	—	10
Brion and Kayser	10	14	9	9	4	2	48
Schottmüller B	12	8	8	5	1	1	35
Aertryck	13	5	4	4	2	—	28
Gaertner L.I.P.M.	12	7	4	13	7	4	47

Typhoid only	35
Typhoid + Brion and Kayser + Schott. B + Aertryck + Gaertner	16
Typhoid + Brion and Kayser + Gaertner	12
Typhoid + Schott. A + Brion and Kayser + Schott. B + Aertryck + Gaertner	10
Typhoid + Brion and Kayser + Schott. B + Gaertner	7
Typhoid + Brion and Kayser	2
Typhoid + Gaertner	1
Typhoid + Schott. B + Aertryck + Gaertner	1
Typhoid + Schott. B + Gaertner	1
Typhoid + Brion and Kayser + Aertryck	1
	86

The fact that typhoid sera may also agglutinate Gaertner bacilli seems to have been first noted by Grüber and Durham (1896), and subsequently developed by Durham (1898 *a*, *b*) who elaborated the correct interpretation of the phenomenon (1901). Pioneer observations were also made by Lorrain Smith and Tennant (1899) who found that, of 123 cases in which the blood of clinical typhoids reacted with typhoid bacilli, 24 per cent. reacted also with Gaertner and 11 per cent. with *Aertryck*[1]. Klein (1903) noted that most typhoid sera would also agglutinate Gaertner to some extent, and Fox (1905 *b*) found Gaertner reactions in 87 per cent. of 94 cases. Zupnik and Poser (1903) found 89 per cent. of 64 typhoid sera to react with paratyphoid B and 40 per cent. with paratyphoid A; Korte and Steinberg (1905) had 56 per cent. and 53 per cent. respectively in 70 cases, and Manteufel (1905) 62 per cent. and 60 per cent. in 85 cases. Exact comparison between these and the present figures is not possible owing to differences in minimal dilutions and standards of reactions.

There is thus considerable variation in the presence, degree, and kind of subsidiary agglutinations in different typhoid sera. The most obvious factor in this variation as regards presence and degree is the typhoid-strength; from the figures already given it appears clear that the more typhoid agglutinin is present, the more subsidiary agglutination is likely to be found. Thus of the 36 sera with a typhoid-strength of less than 1 : 100 only 8 per cent. show coagglutination, while of 50 which rise above that figure 96 per cent. agglutinate also other bacilli. The table shows, however, that marked exceptions to this rule may occur; thus one serum reacting at 1 : 2,000 with typhoid failed to agglutinate

[1] The "*B. enteritidis Hatton*" of Smith and Tennant appears to be a hog-cholera (*Aertryck*) organism. Durham (1898 *b*) found that agglutination tests placed the organism he isolated from the Hatton outbreak with "Günther" (1897) rather than with Gaertner's original strain, and Morgan (1905) has shown that "Günther" is an Aertryck organism.

any of the other organisms at 1 : 20. Others have obtained similar results: thus Manteufel (1905), examining 85 typhoid sera with typhoid, paratyphoid A and paratyphoid B, found coagglutinations present in 56 per cent. of those with a typhoid-strength of 200 or less, and in 78 per cent. of those stronger than this. The great variability in the coagglutination associated with the same typhoid-strength in different sera and in the same serum at different times is, however, sufficient to indicate that a serum does not possess this property of reacting with others than the homologous organism in virtue of its homologous strength alone. It should not be held that typhoid agglutinin is able to produce agglutination of, *e.g.* Gaertner bacilli, if it be present in sufficiently high concentration, but rather that most typhoid agglutinins are possessed of combining groups for other bacilli in quantities which are very variable but (nearly always) far less than for typhoid. It is only when the amount of typhoid agglutinin rises to a fair height that these subsidiary groups become sufficiently numerous to be appreciable by the ordinary methods of examination. That secondary agglutinations are not a function of the strength of the primary agglutinin is also demonstrated by absorption tests: it is found that the secondary agglutination may be entirely removed by the homologous subsidiary organism while the strength of the primary agglutinin is still left above the limits which are commonly associated with the presence of coagglutinations.

The next table of 65 human sera shows that, as the titre for typhoid increases, so the *relative* amount of subsidiary agglutinins diminishes:

Typhoid titre	Number of cases	Average total absolute secondary agglutinins	= per cent. of typhoid agglutinin
50	15	11	23
100	7	69	69
200	6	92	46
500	7	167	33
1,000	11	533	53
2,000	6	645	32
5,000	8	1,196	24
10,000	5	1,978	20

In a general way the increase of typhoid agglutinin accompanies the further progress of the earlier part of the attack of typhoid fever; there is then a tendency to relative purification as the infection persists. This is shown by the following agglutinative histories of four rabbits, which also illustrate the general variability which occurs:

Rabbit I: typhoid *A. E. Wright.*

Time days	Number of inocu-lations	Absolute titre for typhoid	Total[1] absolute secondary agglutinins	Relative (typhoid=100) titre for *Schott. A*	*Schott. B*	*Aertryck*	*Gaertner*	Total
7	2	50	4	4	0	0	4	8
12	2	1,000	160	0	1	5	10	16
21	3	2,000	290	1	1	2·5	10	14·5
56	4	2,000	200	0	0	0	10	10

Rabbit III: typhoid *Lincoln.*

7	2	200	140	1	50	10	10	71
12	2	1,000	160	0	5	1	10	16
21	3	2,000	120	0	2·5	2·5	1	6
56	4	10,000	100	0	1	0	0	1

Rabbit II: typhoid *A. E. Wright.*

Absolute titre for typhoid	Total absolute secondary agglutinins	Relative (typhoid=100) titre for *Schott. A*	*Schott. B*	*Aertryck*	*Gaertner*	Total
200	22	0	5	1	5	11
500	40	0	4	2	2	8
2,000	140	0	1	1	5	7
5,000	70	0	0	0·4	1	1·4

Rabbit IV: typhoid *Lincoln.*

500	160	2	2	10	20	34
1,000	400	1	10	10	20	41
2,000	1,100	1	2·5	2·5	50	56

(dead)

[1] *I.e.* total for those organisms which were used: other secondary agglutinins (*e.g.* for *B. coli*, *B. dysenteriae*) were doubtless present. The minimal dilution in these observations was 1 : 2.

As far then as any temporal relations can be laid down as to the occurrence of secondary agglutinins, it would appear that they are apt to be especially prominent in the early stages of reaction to infection; this absolute and relative abundance is replaced by a stage of relative decrease and absolute increase, which is in turn succeeded by a tendency towards absolute decrease. Fox (1905 *a*, *b*) has especially called attention to the occurrence of Gaertner agglutinins in both human and rabbit typhoid sera before the appearance of the typhoid reaction, and finds that the agglutinative purity of the serum increases as the disease progresses. Park and Collins (1904, p. 505) note this, and also call attention to a marked decrease of purity which may occur after prolonged artificial immunisation.

Other factors are race of infecting organism, and species and individual of infected animal. The former is illustrated by the last table: in the first three weeks, the two typhoid *Lincoln* rabbits produced more than three times the total relative quantity of secondary agglutinins as those inoculated with typhoid *A. E. Wright*, the primary agglutinin being about the same in all four animals. The same animals illustrate the individual variation. With regard to the influence of species, Klein (1904, p. 478) has pointed out that sera of mixed agglutinations are much more readily induced in the rabbit than in the guinea-pig by inoculation of typhoid or Gaertner.

Little need be said as to the kind of secondary agglutinin developed under varying circumstances. It is clear that those for paratyphoid A (*Brion and Kayser*) and Gaertner are the most frequent and generally the stronger: in the series of human sera already given (p. 45), Gaertner is preeminent 19 times, *Brion and Kayser* 9 times, and the two together 13 times. The other type of secondary agglutination among the organisms concerned is much less frequent, and paratyphoid B is in excess but three times. Nothing is clear as to the circumstances which influence this.

Paratyphoid and Gaertner immune sera contain relatively less secondary agglutinins. As the results of typhoid sera suggest, they all react with typhoid freely and usually to a much greater degree than with the heterologous members of their own group. In other words typhoid bacilli have many more combining groups of low specific affinities than have the other varieties of organisms under consideration.

V. *The serum diagnosis of paratyphoid infection.*

The absolute bacteriological diagnosis of paratyphoid fever must involve the isolation of one of the two specific organisms from the stools, urine, pus or, preferably, the blood of the patient, followed by the satisfactory identification of the bacillus by cultural and agglutination tests. Conradi (1904) has shown that paratyphoid, like typhoid, bacilli may occur in the stools of healthy contacts: unless therefore the organism has been found in some part of the body more truly internal than the alimentary tract, the presence also of a state of specific infection must be shown by the serum reactions of the blood. Irregular local paratyphoid affections seem to be at least as common as those caused by typhoid bacilli, appearing either as sequelae to a typhoid-like illness or arising apparently independently of any general infection. In the latter category fall, for instance, the cases recorded by Widal and Nobécourt (1897) who found their "paracolibacille" (= paratyphoid B) in a thyroid abscess, and by Blumenthal (1904) who isolated paratyphoid A from the gall-bladder. Netter and Ribadeau-Dumas (1905) have also recently observed that the sera of a number of cases of acute jaundice agglutinate paratyphoid A (*Brion and Kayser*) to a limit considerably beyond that for typhoid; they suggest that such cases are in reality an "angiocholicystite paratyphique." As examples of the discovery of paratyphoid in local "post-typhoid" suppurations, we may recall the recovery of paratyphoid B from an osteomyelitis by Achard and Bensaude (1896) and Cushing (1900), and from an orchitis as well as from the gall-bladder four years after an attack of "typhoid" by Pratt (1903). In these local infections, the serum reactions are commonly not very well-developed; their rather common occurrence must however be considered in the interpretation of the phenomena observed in the examination of the blood.

Considerations of time and opportunity however must often render complete examinations impossible. It is therefore desirable to consider how far a diagnosis may be formed upon the serum reactions alone.

(*a*) *Agglutinations.* The diagnosis cannot be made on a demonstration that the serum of a patient suffering from an illness resembling typhoid fever fails to agglutinate the *B. typhosus* even in low dilutions (*e.g.* Mackie 1905). Still less is the diagnosis of paratyphoid infection warranted by the bare fact that agglutination is observed with paratyphoid or similar organisms: this is a common property of typhoid and

other sera. Whether a reasonably probable diagnosis can be made after a complete determination of the ultimate limits of agglutination with both paratyphoid and typhoid bacilli is an important practical matter on which opinion is much divided. On the one hand Jürgens (1903, 1904), Drigalski (1904), Grünberg and Rolly (1905), Pratt (1905), Fox (1905 *a*, *b*), Conradi (1904) and Kayser (1904 *a*) are, among other observers, very sceptical as to the value of agglutinations in the differential diagnosis of typhoid and paratyphoid, while of recent authors Korte and Steinberg (1905), Manteufel (1904) and Zupnik (1905) believe that the method gives on the whole accurate results. Pratt (1903), for example, records a case of "typhoid" where the serum reacted with paratyphoid B at 1 : 200 and gave no reaction with typhoid at 1 : 10; blood cultures gave a pure culture of *B. typhosus*. Fox (1905 *a*) obtained *B. typhosus* from the blood of a patient whose serum agglutinated paratyphoid A at higher dilutions than typhoid throughout the illness; the difference was most marked during a relapse. The same author (1905 *b*) notes that some reaction with Gaertner was present in 82 of 94 typhoid sera, and that in about a dozen cases this secondary agglutination appeared earlier in the disease than, or exceeded the limit of, the typhoid reaction. The results of Grünberg and Rolly (1905) are even more remarkable: of 40 cases of undoubted clinical typhoid, 32 gave *B. typhosus* on blood culture; coagglutinations were present in 28 and in 14 the reactions with paratyphoid A or Gaertner occurred at higher dilutions than with typhoid. Such results are in strong contrast with those of Korte and Steinberg (1905) and Manteufel (1905) who examined respectively 70 and 85 typhoid sera with *Schottmüller A* and *B* and found no case where the agglutination limits for these surpassed the limit for typhoid. In the present series of 176 typhoid sera, the secondary agglutinations were never observed to be in excess of the primary reaction: in two instances the limits ascertained for typhoid and paratyphoid A (*Brion and Kayser*) were the same, though it should be noted that the limits reached were only 1 : 50 and 1 : 100. A later sample of serum from one of these cases gave typhoid = 1 : 100, *Brion and Kayser* = < 1 : 20: the other could not be further examined.

In a valuable contribution dealing with 300 cases of typhoid, Zupnik (1905) records only the following instances of irregularity in the agglutination results:

B. typhosus isolated from	Agglutination limits for		
	Typhoid	*Schottmüller B*	*Brion and Kayser*
Spleen	80	200	0
Blood	0 (<40)	80	0[1]
Faeces	1,600	120	2,000
Spleen	80	0	80
Urine	200	200	0
Blood	200	40	200[1]

He points out that equality or inferiority of typhoid agglutinin is likely to be less frequent the greater the number of races of typhoid used for the tests; further that, as far at any rate as paratyphoid B is concerned, *approximate or complete equality of typhoid and paratyphoid agglutination limits indicates a typhoid infection.* In 21 cases of infection with paratyphoid B, the least difference between the paratyphoid and typhoid agglutination was in a case where the ratio of the two was 6·2 : 1, the average ratio being about 240 : 1. In none of the recorded cases has the superiority of paratyphoid over typhoid in a typhoid serum been of the same order of magnitude. In this connection the high absolute figures often obtained with paratyphoid B sera may be noted: generally these are far beyond the values obtained for typhoid in typhoid sera. Thus Savage (1905, p. 344) notes a reaction of 1 : 70,000, my own cases vary from 1 : 5,000 to 1 : > 20,000, and of Zupnik's (1905, p. 1751) 21 patients only one was less than 1 : 1,000, nine exceeded 1 : 10,000 and three reached the remarkable figures of 1 : 96,000, 1 : 128,000 and 1 : 140,000.

The opinion that the results of agglutination experiments are unreliable is to some extent founded on errors of technique. Thus Drigalski (1904) found that in 9·3 per cent. of 257 cases the agglutination of paratyphoid B was equal to, and in 10·1 per cent. more than, that of typhoid. The conclusion that the method is unreliable is altogether invalidated by the fact that the observations were only made at dilutions of 1 : 50 and 1 : 100, no attempt being made to ascertain the *ultimate limits* of agglutination for the two organisms. Attempts to measure the amount of agglutinin by the apparent completeness of the reaction at a low dilution result only in an increase of the errors which are inherent to the method under the most favourable circumstances.

Other sources of error may occur in the organisms used for agglutination. Three somewhat striking examples of the failure of an animal serum to identify its homologous bacillus have been met with in the course of the present investigation. The first illustrates the importance

[1] Later examinations of these cases showed a higher limit for typhoid.

of ascertaining the conditions of growth of the test cultures best suited for agglutination experiments.

Rabbit: inoculated with *Lincoln* typhoid four times in a period of 29 days: agglutination limits of serum:

Conditions of growth of test culture	Room 17 hrs.	36° 17 hrs.	43° 17 hrs.	43° 3½ hrs.
Typhoid *Guy's*	2,000	2,000	2,000	2,000
,, *Lincoln*[1]	1,000	100	100	<50
Schottmüller A	—	20	—	—
,, *B*	—	50	—	—
Aertryck	—	50	—	—
Gaertner L.I.P.M.	—	500	—	—

The second instance illustrates the difficulties which are raised by an infection with an organism which, though agglutinogenic, is relatively non-agglutinable. Such was the condition of the strain of *Schottmüller A* used in these experiments. Two rabbits were inoculated repeatedly with this organism: their sera gave the following results:

	Agglutination limits with					
	Typhoid *Guy's*	*Schott. A*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*
Rabbit A: 27. vi. '05	1,000	100	—	20	20	200
Rabbit B: 24. vii. '05	100	10	—	10	<10	10
1. viii. '05	200	20	<20	<20	20	20
15. viii. '05	500	100	2,000	500	500	500

In the first case the use of a culture grown at about 17° instead of 36° brought the agglutination results in correspondence with the known origin of the serum. In the second instance however no such procedures produced any material modification in the agglutinability of the homologous organism; the use of another strain (*Brion and Kayser*) of the same bacillus was here necessary.

The third example of such errors is of the opposite nature. The culture of *Brion and Kayser* in our possession, when emulsified in any mixture of normal or immune serum at any dilution, shows a remarkable tendency to spontaneous sedimentation. So much so, that, after two hours in the hot incubator, the macroscopic agglutination is in nearly all cases practically complete, though microscopically in the hanging drop there may be no reaction at all. The use of such an organism in the macroscopic way would lead to the most erroneous conclusions, and it is perhaps not insignificant that many of the results, which are

[1] It is possible that this phenomenon is a partial explanation of the lack of agglutinability commonly ascribed to recently-isolated typhoid strains. The old laboratory strain (*Guy's*) gave the same results under all conditions.

considered to condemn the use of agglutination phenomena for the differential diagnosis of typhoid and paratyphoid infections, have been obtained by the macroscopic method.

Korte and Steinberg (1905) have called attention to another possible source of fallacy arising out of the "zone of inhibited agglutination." In low dilutions, a typhoid serum may fail to agglutinate typhoid bacilli, giving at the same time a good reaction with paratyphoid; at higher dilutions the typhoid reaction appears, and ultimately far surpasses that with paratyphoid. It is interesting to note that the authors found this phenomenon to be materially misleading only when the macroscopic method was used; microscopical controls revealed the real nature of the serum without much difficulty.

On the whole then, it would appear that an accurate determination of the ultimate limits of agglutination, preferably by the microscopic method, with organisms of known agglutinable capacity, and with cultures grown under predetermined optimum conditions, will, at any rate in the great majority of cases, give accurate information as to the nature of a typhoid or paratyphoid serum. At the same time it must be allowed that anomalous results are occasionally obtained, especially in the early stages of infection, and a positive diagnosis of paratyphoid should not be lightly made since doubtful cases are usually typhoid.

(*b*) *Absorption tests and mixed infections.*

The determination of the agglutination values of the different organisms reacting with any given serum will not, however, exclude the presence of a double or multiple infection. Such mixed infections are necessarily uncommon in so far as typhoid, paratyphoid and similar organisms are concerned. The incidence of clinical typhoid fever in London varies but little from 0·07 per cent.; paratyphoid fever can hardly be more frequent than 0·005 per cent.: the coincidence of the two diseases should therefore be of almost infinite rarity. There is however little reason to doubt that paratyphoid is disseminated by the same excretory contamination, direct and indirect, which is responsible for the spread of typhoid. This similarity must considerably reduce the mathematical improbability of their coincidence. Typhoid and paratyphoid organisms have in fact been isolated from the same person by several observers (Conradi 1904, Kayser 1904 *a*: see also Savage 1905, p. 349), though such instances are in part no doubt cases of para-

typhoid infection in an individual harbouring, but not in a state of infection by, typhoid organisms (or *vice versâ*).

For the diagnosis of mixed infections, absorption experiments ("Castellani's test") are of the first importance. Castellani (1902) found that excess of typhoid bacilli would remove not only the primary (homologous) typhoid, but also the secondary (heterologous) *coli*, agglutinins from the serum of a rabbit inoculated with typhoid, while from a serum giving similar agglutinations (at any rate qualitatively) but elaborated in response to infection with *B. coli* as well as *B. typhosus*, neither typhoid nor *coli* bacilli alone, but only both together, simultaneously or successively, would remove the whole of the agglutinin.

The accuracy and value of the test is generally acknowledged; some illustrative examples of single and double infections are given here. The technique used has been already explained (p. 37).

Nature of serum	Dilution[1]	Absorbed with	Agglutination (after absorption) with: Typhoid *Guy's*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*	*Schott. A*
I. Typhoid: human	1 : 10	Original titre	2,000	1,000	50	50	500	50
		Typhoid Guy's	+ + +	0	0	0	+ + +	—
			0	0	0	0	0	—
		Brion and Kayser[2]	+ + +	+ +	0	0	+ + +	—
			+ + +	0	0	0	+ + +	—
		Schott. B	+ + +	+ +	0	0	+ +	—
			+ + +	0	0	0	+ +	—
		B. coli communis[3]	+ + +	+ +	+ + +	+ +	+ + +	—
II. Paratyphoid B: human: case "Barkley" 31. vii. '05: see also p. 64.	1 : 10	Original titre	200	500	>5,000	1,000	200	50
		Typhoid Guy's	0	+ + +	+ + +	+ + +	+	—
		Brion and Kayser	+ +	+ + +	+ + +	+ + +	+ + +	—
			+ +	0	+ + +	+ + +	+ + +	—
		Schott. B	0	0	+ + +	+ + +	0	—
			0	0	+ + +	+ + +	0	—
			0	0	0	0	0	—
		Aertryck	0	0	+ + +	0	0	—
		Gaertner L.I.P.M.	0	+ + +	+ + +	+ + +	0	—
		B. coli communis	+ + +	+ + +	+ + +	+ + +	+ + +	—
		Guy's + Brion and Kayser + Aertryck + Gaertner	0	0	+ + +	0	0	—

[1] The agglutinations were observed at twice this figure.

[2] These two series of figures under one heading indicate the results of successive additions of absorbing bacilli and show to some extent the order of removal of the different secondary agglutinations. Thus after the first extraction of the serum with *Brion and Kayser* the remainder agglutinated *Guy's*, *Brion and Kayser* and *Gaertner*; after a second extraction, *Guy's* and *Gaertner* still reacted and the *Brion and Kayser* agglutination failed (*i.e.* the end point of this absorption was reached).

[3] The original Escherich strain.

Nature of serum	Dilution	Absorbed with	Agglutination (after absorption) with: Typhoid *Guy's*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*	*Schott. A*
III. Gaertner original A: rabbit	1 : 25	Original titre	1,000	200	20	<20	5,000	50
		Typhoid Guy's	0	0	0	—	+ + +	—
		Brion and Kayser	+ +	0	0	—	+ + +	—
		Schott. B.	+ + +	0	0	—	+ + +	—
		Gaertner L.I.P.M.	0	0	0	—	0	—
		Aertryck	+ + +	0	0	—	+ + +	—
		B. coli	+ + +	+	0	—	+ + +	—
IV. Aertryck: rabbit	1 : 50	Original titre	200	500	2,000	2,000	20	—
		Typhoid Guy's	0	+ + +	+ + +	+ + +	—	—
		Brion and Kayser	0	0	+ + +	+ + +	—	—
		Schott. B	0	+	0	+ + +	—	—
		Aertryck	0	0	0	0	—	—
V. Typhoid *Delépine* + Schottmüller B: rabbit	1 : 50	Original titre	1,000	2,000	5,000	200	<20	—
		Typhoid Guy's	0	0	+ + +	+ +	—	—
		Brion and Kayser	+ + +	0	+ + +	+ +	—	—
		Schott. B	+ + +	0	0	0	—	—
		Typhoid + Schott. B	0	0	0	0	—	—
		Schott. B + Brion and Kayser	+ +	0	0	0	—	—
		Typhoid + Brion and Kayser	0	0	+ + +	+ +	—	—
VI. Typhoid *A. E. Wright* + Brion and Kayser: rabbit	1 : 25	Original titre	5,000	1,000	100	20	200	—
		Typhoid Guy's	0	+ + +	—	—	0	—
		Brion and Kayser	+ + +	0	—	—	+ + +	—
		Gaertner L.I.P.M.	+ + +	+ + +	—	—	0	—
		Gaertner + typhoid	0	+ + +	—	—	0	—
		Gaertner + Brion and Kayser	+ +	0	—	—	0	—
		Typhoid + Brion and Kayser	0	0	—	—	0	—
VII. Schottmüller A: rabbit	1 : 25	Original titre	500	2,000	500	500	500	100
		Typhoid Guy's	0	+ + +	+ + +	+ + +	0	0
		Schott. A	0	0	+ +	+ +	—	—
		Brion and Kayser	0	0	+ + +	+ +	—	0
		Schott. B	0	+ + +	0	0	—	0
		Aertryck	0	+ + +	0	0	—	+

These figures require little comment. Example IV illustrates the diagnostic import of absorption when the agglutination values for the homologous and a heterologous organism are approximately identical. Example VII shows the irregular results obtained in dealing with an organism of bizarre agglutinative character (see p. 54). Apart from

this, no instance has been noted in which the absorption results have been demonstrably misleading, though indications have been obtained which suggest that Castellani's rule may not always hold good if the subsidiary organisms are widely heterologous to the reacting serum (see below p. 68). Opportunity has however been lacking for experimentation with sera of irregular agglutination. In most cases the results of absorption follow those of agglutination, and the two phenomena are essentially the same. They differ however in that absorption is conditioned by the total quantity of available combinations, while the immediate ocular demonstration of this reaction depends on the quantity of such combinations in immediate connection with the bacillary bodies. The occurrence and varying relative abundance of receptors free in the circumjacent fluid[1] are important factors over which we have imperfect control: they probably account for some of the irregularities observed.

Jürgens (1903, 1904) has described the absorption phenomena with typhoid sera of irregular agglutination limits. A typical example gave

	Agglutination limits with	
	Typhoid	Paratyphoid B
Normal	50	800
Absorbed with typhoid	0	300
,, ,, para. B	50	100

and is interpreted as being indicative of typhoid infection on account of the failure of paratyphoid B bacilli to remove any of the typhoid agglutinin. It is however difficult to understand how this could happen if the typhoid bacilli were able to remove more than half the paratyphoid agglutinin. My own experiments, as far as they cover the same ground, are not in accordance with this observation. They show that the primary agglutinin is absorbed by saturation with the bacilli of the secondary agglutinins, and the secondary agglutinins by the bacilli of one another, in amounts which are in a general way proportional to the quantities of secondary agglutinin present, though some allowance has to be made for the varying degree to which the combining groups of the different bacilli are mutually available[2]. For example:

[1] See M. Neisser and K. Shiga (*Deutsche medizinische Wochenschrift*, 1903, p. 61) and B. H. Buxton and J. C. Vaughan, Jr. (*Journal of Medical Research*, 1904, vol. XII. p. 138).

[2] Without entering at length on a complex subject, it may be briefly stated that these mutually combining groups appear from absorption experiments to be arranged in the same way as would be expected from the agglutination reactions. Thus *Brion and Kayser*, and *Aertryck* are more combinable, both mutually and with typhoid, than with paratyphoid B or Aertryck; this reproduces the arrangement of the frequency and intensity of the secondary agglutinins in typhoid and paratyphoid sera (p. 45). The hypothesis of Durham (1901) affords the best terms in which to express the phenomena.

Rabbit: typhoid (*A. E. Wright*) immune serum.

	Agglutination limits with			
	Typhoid *Guy's*	*Schott. A*	*Schott. B*	*Gaertner L.I.P.M.*
Before absorption	2,000	0	20	200
Absorbed with Typhoid *Guy's*	500	0	0	0
,, ,, *Schott. A*	2,000	0	20	—
,, ,, *Schott. B*	2,000	0	0	—
,, ,, *Gaertner L.I.P.M.*	500	0	20	0

Park and Collins (1904, p. 498) have called attention to the fact that absorption experiments may give erroneous results in mixed infections unless the bacteria concerned have been already suggested by agglutination or other phenomena. The paratyphoid A (*Brion and Kayser*) agglutinin, for example, found in the serum (number V above) of a typhoid infection may be completely removed by saturation with typhoid bacilli, and may be due in large part or altogether to an unsuspected concurrent infection with paratyphoid B. Its true nature would not appear unless the paratyphoid B infection had been already suggested and absorption tests with paratyphoid B bacilli made in consequence. "The absorption method simply proves, therefore, that when one variety of bacteria removes all agglutinins for a second, that the agglutinins under question were not produced by that second variety[1]."

(c) *Variation in type of agglutination.*

Attention has already been drawn to the fact that the "clumps" formed by Gaertner, paratyphoid B or Aertryck bacilli with homologous sera are relatively loose aggregations and of very different appearance to the dense masses seen in the reaction of typhoid bacilli with typhoid serum. The same form of reaction is commonly seen when any one of these three organisms reacts with a serum homologous with either of the other two. It is of some theoretical interest and of considerable practical diagnostic value to note that, when typhoid serum reacts with these organisms in a heterologous way, the clumps follow the typhoid type and appear as tight masses. The same, but to a less degree, is true for paratyphoid A: thus, with a *Brion and Kayser* rabbit serum, *Brion and Kayser* often forms loose masses (though frequently tight clumps) while paratyphoid B, Gaertner and *Aertryck* form typhoid-like clumps, as does *Brion and Kayser* in typhoid serum. To typhoid bacilli

[1] These considerations render the diagnosis of the much discussed *coli* infections in typhoid fever and similar conditions rather complicated owing to the marked specificity of the agglutinins of different races of *B. coli* (van Everen 1905).

the phenomenon does not apply; the clumps in homologous and heterologous sera are not markedly dissimilar.

These differences are entirely independent of the concentration of the serum and, as far as the cultures at my disposal are concerned, of the race of organism. Their common usefulness is found in the routine examination of typhoid sera in low dilutions where the typhoid-like clumps of the Gaertner reaction which is so often present suggest at once that this is a secondary, rather than a primary, agglutination.

(*d*) *The thermostability of paratyphoid agglutinins.*

It is well-known that agglutinins are relatively thermostabile, *i.e.* they are not rendered inactive by heating at 60° C. for 30 minutes. The agglutinins for Gaertner and paratyphoid have been found to be relatively more heat-resistant than those for typhoid. In these experiments the sera under comparison were mixed and then heated successively at 65°, 70°, 75°, etc. in a water-bath for 30 minutes at each temperature. A typical observation on a mixture of four immune rabbit sera gave the following agglutination limits:

	Typhoid *Guy's*	*Brion and Kayser*	*Schott. B*	*Gaertner L.I.P.M.*
Before heating	10,000	10,000	500	500
Heated at 70°	5,000	10,000	500	500
,, ,, 75°	<20	5,000	200	200
,, ,, 80°	—	500	<20	20
,, ,, 85°	—	<20	—	<20

The next table gives the average results of six such experiments with sera of various concentrations; the numbers represent the agglutination limits after heating expressed as percentages of the limits found in the unheated sera:

	Absolute limits before heating					
	Max.	Min.	Unheated	70°	75°	80°
Typhoid	10,000	500	100	22	0	0
Gaertner	20,000	500	100	83	32	6
Paratyphoid B	5,000	500	100	55	30	0
Brion and Kayser	10,000	1,000	100	63	45	4½

In such experiments it seems to be immaterial (within the limits of error of the method) whether the serum is slightly (1 : 10) diluted and then heated, the necessary further dilutions being made subsequently, or whether the serum is fully diluted before heating. In some instances the disappearance of agglutinin seems to be somewhat greater in the former circumstances, in others in the latter. The results are, however,

naturally different if the serum tested at one temperature has been previously exposed to a lower temperature, so long, that is, as this latter be above the lower limit at which destruction[1] begins to be manifest in periods of time comparable to 30 minutes. It should be clearly recognised that the destruction by heat of these properties of serum is not instantaneous but a gradual process, occupying at temperatures in the neighbourhood of 60° periods of time such that the conventional half-hour can in no way be regarded as infinite. It is a function of time as well as of temperature, so that the absolute amount destroyed at any given temperature is conditioned by the amount originally present if the time-limit falls materially short of that period in which the rate of destruction by that temperature falls almost to zero. The apparent effect of any heating is then distinctly different with sera of different strengths. If, for example, a certain combination of time and temperature reduces the agglutination limit by 95 per cent., the residues of two sera of titres 5000 and 100 will be 250 and 5 respectively. In the former case the agglutinin is manifestly only reduced, in the latter it is commonly spoken of as being destroyed. There are, however, certain temperature limits below which the times necessary to destroy any considerable quantity of agglutinin are very long relatively to 30 minutes. Using such a short period of heating, it is then perhaps permissible to speak of the destruction beginning and ending at certain points. From the results here obtained it would appear that this destruction of typhoid agglutinin commences at about 58° and is complete at about 75°, the corresponding figures for Gaertner agglutinin being 65 + ° and 83°. The coarse methods of measurement used tend however to obscure the earlier phases and to place the apparent point of commencing destruction somewhat higher in ordinary practice. It follows that 30 minutes at 65° *plus* 30 minutes at 70° will affect a Gaertner agglutinin to much the same degree as one heating at 70°: the latter will however have the smaller effect on a typhoid agglutinin.

The practical application to diagnosis of the differences in thermostability is rendered somewhat difficult by the preponderating influence of the quantities of agglutinin subjected to examination, as well as by the fact that different sera yield rather varying results even when allowance is made for their different concentrations[2]. It lies

[1] A convenient mode of expression and perhaps justifiable: *qua* the phenomenon of agglutination, agglutinins are destroyed by conversion into agglutinoids.

[2] It is important in practice to see that the serum to be heated is free from red cells and haemoglobin. Heating these is liable to produce a very fine precipitate which cannot be

however in the observation that *any Gaertner or paratyphoid agglutinin found in a typhoid-immune serum disappears at a lower temperature than the primary typhoid agglutinin.* Differences of concentration may be the whole explanation of the abolition of the Gaertner reaction at a lower, rather than at the same, temperature, though it would appear that secondary agglutinins generally are distinctly less thermostabile than the primary agglutinins. Thus a Gaertner-immune serum giving Gaertner = 20,000 and typhoid = 5,000 showed, after heating for 30 minutes at 60°, Gaertner = 20,000 and typhoid = 100, while typhoid-immune sera of comparable strength under similar circumstances show a loss of less than 50 instead of more than 90 per cent.

It is perhaps germane to this subject to recall the fact that the toxins of Gaertner and paratyphoid are notoriously heat-resistant. A remarkable elevation of the thermal death-point of the organisms themselves has also been described by B. Fischer (1903, p. 289) who found that half-an-hour at 60° and even five minutes at 75° did not kill all the bacilli of a strain of paratyphoid B isolated by himself in an epidemic at Kiel. This observation I have not been able to confirm; young broth cultures of typhoid (two strains), paratyphoid A (two strains), paratyphoid B (four strains), Aertryck and Gaertner (two strains) all survived 10 minutes at 55° but were as uniformly killed by an exposure of similar length at 60°. Nor does the agglutinable substance of Gaertner bacilli appear to be more heat-resistant than that of typhoid. In my own experiments indeed it seems to be less thermostabile: heating broth cultures of *Gaertner L.I.P.M.* at 65° for 30 minutes greatly reduces or almost abolishes their agglutinability with both Gaertner and typhoid immune sera, while typhoid *Guy's* is far less affected. The results of one such experiment appears in the next table.

It would appear, then, that a good diagnosis may in most instances be made from the examination of the serum alone as to the limits and morphology of its agglutinins, their relation to heating and their combinations with bacillary bodies. Jürgens (1904) has shown that useful results in doubtful cases may be obtained by the direct estimation of the protective power of the serum in the peritoneal cavity of animals. I have not made any observations of the same

removed by filtration or centrifugalisation. This applies especially to human blood; it is not nearly so marked in rabbit blood.

kind, and Böhme (1905) shows that the reaction is not highly specific among members of this group of organisms.

Broth cultures 20 *hours: mixture of typhoid* A. E. Wright, *paratyphoid A* Brion and Kayser, *paratyphoid B* Schottmüller B, *and* Gaertner original A *immune rabbit sera.*

		Typhoid *Guy's*	*Brion and Kayser*	Paratyphoid B *Potts*	*Gaertner L.I.P.M.*
Before heating	1 : 50	+ + +	+ + +	+ + +	+ + +
	1 : 500	+ + +	+ + +	+ + +	+ + +
	1 : 1,000	+ + +	+ + +	+ + +	+ + +
	1 : 5,000	+ +	+ +	+	+ +
Heated at 60° for 30 mins.	1 : 50	+ + +	+ + +	+ + +	+ + +
	1 : 500	+ + +	+ + +	+ + +	+ + +
	1 : 1,000	+ + +	+ + +	+ + +	+ + +
	1 : 5,000	+ +	+ +	+	0
Heated at 70° for 30 mins.	1 : 50	+ + +	+ +	+	+ +
	1 : 500	+ + +	+ +	+	0
	1 : 1,000	+ +	+ + +	0	0
	1 : 5,000	+ +	+	0	0

VI. *Details of cases observed.*

Case I. "*Potts*" (28. iii. '05). Male 8 years. Clinically mild typhoid; onset sudden: duration of fever three weeks. During the first week the blood was examined twice; on both occasions it gave complete agglutination with *Schottmüller B*, but no reaction with typhoid or *Schottmüller A*, at 1 : 50 (higher dilutions not tried). In early convalescence organisms corresponding culturally with *Schottmüller B* were easily obtained by direct plating of hard scybalous stools, both from the surface and the interior of the faecal masses. A third sample of blood obtained a month after the fever had ceased gave the following agglutination limits:

Typhoid *Guy's*	100	*Gaertner L.I.P.M.*		<100
Schottmüller A	<100	Organisms isolated from stools	No. 8	10,000
Schottmüller B	20,000		No. 12	20,000
Aertryck	20,000		No. 29	10,000

Case II. "*Preston*" (14. vi. '05). Male $1\frac{10}{12}$ years. Clinically indefinite but cerebral symptoms predominated and patient was thought to have "meningitis or influenza with nervous symptoms." Onset gradual. Duration about $2\frac{1}{2}$ weeks; contracted varicella in fourth week. At the end of the second week serum completely

agglutinated *Schottmüller B* at 1 : 1,000 but was negative with typhoid and *Schottmüller A* at 1 : 50. From the stools nothing resembling paratyphoid could be obtained by plating direct or after preliminary incubation in dextrose-bile-salt peptone water: after incubation in dulcite medium a fair number of colonies were obtained corresponding culturally with *Schottmüller B*. A second sample of blood obtained in the fourth week agglutinated *Schottmüller B* and the organisms obtained from the stools up to 1 : 10,000.

Case III. "*Barkley*" (28. vii. '05). Male 13 years. Clinically mild typhoid; onset gradual, commencing while patient was in Dublin. The blood examinations gave:

	Typhoid *Guy's*	Typhoid *Lincoln*	*Schott. A*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*
8th day of disease	100	—	50	—	>1,000	500	50
11th ,, ,,	200	200	50	500	>5,000	1,000	200

Absorption experiments with the two samples gave the same results; the details of the first sample are as follows; those of the second being given on p. 56.

	Agglutinations (after absorption) at 1 : 20 with				
Absorbed at 1 : 10 with	Typhoid *Guy's*	*Schott. A*	*Schott. B*	*Aertryck*	*Gaertner*
Typhoid *Guy's*	0	0	+ + +	+ + +	0
Schottmüller A	0	0	+ + +	+ + +	0
,, *B*	0	0	0	0	0
Aertryck	0	0	+ + +	0	0
B. coli communis	+	tr	+ + +	+ + +	+ +

From a specimen of faeces during the third week a single colony corresponding culturally to *Schottmüller B* was obtained after preliminary incubation in dulcite peptone water; many were obtained from the blood of guinea-pigs killed by the intraperitoneal inoculation of dulcite cultures of the stools. In a second sample of stools obtained in the fifth week no organisms resembling paratyphoid could be found.

It will be convenient to describe here together the agglutination reactions of the organisms isolated from these three cases with sera derived from rabbits inoculated with known cultures:

Serum	Typhoid *Guy's*	*Schott. A*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*	*Potts*	*Preston*	*Barkley*
Schott. B	200	50	5,000	200	200	2,000	5,000	5,000
Aertryck	200	0	100	5,000	0	50	100	50
Gaertner original A	1,000	50	0	0	5,000	0	0	0

These results show that the organisms obtained belong clearly to the paratyphoid B (*Schottmüller B*) rather than to the *Aertryck* (hog-cholera) or Gaertner (food-poisoning) group.

Their pathogenicity was typically of a high order. Guinea-pigs of about 250 gms. were inoculated with the following amounts of 20 hours' broth cultures incubated at 37° C.:

	Potts		*Preston*		*Barkley*	
	Subcut.	Intraperit.	Subcut.	Intraperit.	Subcut.	Intraperit.
1 c.c.	dead < 18 hrs.	—	dead 40 hrs.	—	—	—
0·5 c.c.	—	—	—	—	lived	dead < 18 hrs.
0·1 c.c.	dead 40 hrs.	dead < 18 hrs.	dead 12 days *	dead < 18 hrs.	dead 7 days	dead < 18 hrs.
0·01 c.c.	dead 20 hrs.	dead 20 hrs.	dead 40 hrs.	dead < 18 hrs.	dead 50 hrs.	lived
0·001 c.c.	—	dead 28 hrs.	—	—	lived	dead 42 hrs.

* Abscess at site of inoculation; paratyphoid B recovered from heart blood.

Feeding experiments were negative. Two guinea-pigs were fed twice with green food soaked in broth cultures of *Potts*, and two rats and two mice lived for some days on the organs of guinea-pigs killed by inoculation with the same organism. Though the organisms were found in abundance in the faeces of the guinea-pigs a week later, no symptoms of illness were observed, and all the animals were alive and well after two months.

Case IV. *Valérie* (1. viii. '05). Female 45 years. Clinically somewhat atypical typhoid; duration three weeks. The blood was examined in the first, second and third weeks:

	Agglutination limits with								
	Typhoid *Guy's*	Typhoid *Lincoln*	*Schott. A*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*	*Valérie* 21 (see below)	*Valérie* 25 (see below)
1. viii. '05	1,000	—	0	1,000	5,000	—	1,000	—	—
8. viii. '05	500	1,000	0	200	5,000	200	100	—	—
15. viii. '05	1,000	2,000	0	1,000	> 20,000	1,000	200	1,000	0

These results indicate fairly clearly a paratyphoid B infection. The absorption experiments gave however anomalous results; with the second sample the whole of the agglutinins could not be exhausted with typhoid *Guy's*, *Brion and Kayser*, *Schottmüller B* or *Aertryck*. The details for the third sample are given, the chief combinations having been confirmed by absorption in other dilutions:

Absorbed at 1 : 50 with	Agglutinations (after absorption) at 1 : 100 with							
	Typhoid *Guy's*	Typhoid *Lincoln*	*Brion and Kayser*	*Schott. B*	Parat. B *Potts*	*Aertryck*	*Gaertner L.I.P.M.*	*Valérie*
Typhoid *Guy's*	0	0	+ +	+ + +	—	+ + +	0	—
Typhoid *Lincoln*	0	0	0	+ + +	—	+ + +	0	—
Brion and Kayser	0	+ +	0	+ + +	—	+ + +	0	+ + +
Schottmüller B	0	0	+ + +	0	—	0	0	+ + +
Paratyphoid B, *Potts*	0	0	+ +	0	0	0	0	+ +
Aertryck	0	+ + +	+ +	+ + +	—	0	0	—
Gaertner L.I.P.M.	0	+ +	+ + +	+ + +	—	+ + +	0	—
B. coli communis	+ + +	+ + +	+ + +	+ + +	—	+ + +	0	—
Valérie 21	0	+ +	0	+ + +	+ + +	+ + +	0	0
Schottmüller B + Brion and Kayser	0	0	0	0	0	0	0	+ + +

Disregarding the results as far as they concern *Valérie* 21, it would appear from this table that the case was one of mixed infection with paratyphoid A and B, or with paratyphoid B and some unknown organism giving rise to secondary agglutinins for *Brion and Kayser* (see above p. 59).

The stools were examined on two occasions for paratyphoid organisms but none could be found by any method. Two varieties of dulcite-fermenting, non-lactose-fermenting "*coli*-like" organisms were however present in some numbers and require mention.

(*a*) "*Valérie* 25": actively motile; no liquefaction of gelatin; fair quantity of indol; milk strongly acid, but unclotted after 19 days;

acid and gas produced in	no apparent change in
dextrose	*lactose*
laevulose	*cane-sugar*
maltose	raffinose
galactose	*sorbite*
arabinose	erythrite
dextrin	amygdalin
mannite	inulin
dulcite	
salicin	

At first sight this organism is very similar to *Schottmüller A* or *Brion and Kayser*. It differs however culturally from these organisms in its rapid and extensive fermentation of salicin and in its failure to attack sorbite. With the serum of rabbits inoculated with *Schottmüller A* or *Brion and Kayser* it shows no agglutination even in low dilutions, and the cultures from the stools of the present case entirely failed to

react with the patient's, or indeed any other, serum at 1 : 20. The same organism was isolated in large numbers from the urine and faeces of the next case (*Shepherd* 1). The pathogenicity was not high; a 200 grm. guinea-pig survived the intraperitoneal injection of 1 c.c. of a 24 hours' broth culture of *Valérie* 25; of *Shepherd* 1 0·5 c.c. killed in 18 hours, but 0·1 c.c. was not fatal. This bacillus appears indeed to be very close to the "B. paratyphoid A, unknown type," obtained by Morgan (1905, p. 1260) from the faeces and intestines of animals and from milk, and is probably a not uncommon intestinal organism. Dr Morgan has been good enough to furnish me with a culture of his bacillus; I find that it ferments salicin readily and fails to attack sorbite in 48 hours; acid and gas is however produced in 7 days. Its pathological relations are unknown; possibly it has none. It is however of interest as bearing a somewhat deceitful resemblance to paratyphoid A.

(β) "*Valérie* 21": actively motile; no liquefaction of gelatine; fair quantity of indol; milk rapidly acidified and clotted;

acid and gas formed in	no apparent action on
dextrose	*lactose*
laevulose	erythrite
maltose	*salicin*
galactose	amygdalin
cane-sugar	inulin
arabinose	
raffinose	
dextrin	
dulcite	
mannite	
sorbite	

A 200 grm. guinea-pig died overnight after receiving 0·1 c.c. of a 24 hours' broth culture intraperitoneally. This organism has several radical cultural distinctions from the paratyphoid group, and only finds mention here on account of its agglutinative reactions. With the patient's serum (15. viii. '05) it gave a complete reaction up to 1 : 1000; after admixture with excess of this bacillus, the serum lost not only this reaction but also failed to agglutinate *Brion and Kayser*, the agglutinin for which could not be removed by the organism (*Schottmüller B*) which was predominant in the agglutinations. On the other hand, the reaction

with *Valérie* 21 was not removed by treatment with *Schottmüller B* and *Brion and Kayser* which together removed all the other agglutinins[1]. These results suggest that the condition in the present case was possibly one of double infection with paratyphoid B and *Valérie* 21, the latter being the source of the *Brion and Kayser* agglutination, and the former having secondary agglutinins for *Aertryck* and typhoid *Lincoln*. The phenomena however do not necessarily imply that *Valérie* 21 had any aetiological connection with the present case. It seems to the writer that they are otherwise explicable on the grounds (1) that the organism in question is one of very high agglutinability; it has in fact been found that it agglutinates at 1 : 200 to 1 : 500 with *Schottmüller B*, *Brion and Kayser*, *Aertryck* and typhoid rabbit sera (agglutinating their homologous bacilli at 1 : 1,000 to 1 : 10,000), showing at the same time no trace of agglutination in normal saline: (2) that such an organism, differing widely in its cultural reactions from the group to which the sera belong, may not altogether conform to Castellani's rule in absorption experiments. As an example its behaviour in the serum of a rabbit inoculated with *Schottmüller B* may be quoted:

Absorbed at 1 : 10 with	Agglutination (after absorption) at 1 : 20 with							
	Typhoid *Guy's*	Typhoid *Lincoln*	*Brion and Kayser*	*Schott. B*	Parat. B *Potts*	*Aertryck*	*Gaertner L.I.P.M.*	*Valérie* 21
Typhoid *Guy's*	0	0	+ + +	+ + +	+ + +	+ +	0	+ +
Brion & Kayser	+	0	0	+ + +	+ + +	+ + +	0	+ +
Schott. B	0	0	0	0	0	0	0	+ +
Valérie 21	+ +	+	+ + +	+ + +	+ + +	+ + +	0	0
Original titre	1,000	500	5,000	10,000	10,000	200	50	200

On the other hand the failure of this organism to conform to the rules of absorption does not always hold good. With a *Brion and Kayser* rabbit serum the following results were obtained:

Absorbed at 1 : 25 with	Agglutination at 1 : 50 with		
	Typhoid *Guy's*	*Brion and Kayser*	*Valérie* 21
Typhoid *Guy's*	0	+ + +	+ + +
Brion and Kayser	0	0	0
B. coli	0	+ + +	+ +
Original titre	500	1,000	100

The exact nature of this case remains uncertain. The high agglutination value of the third sample of serum with *Schottmüller B* (1 : 20,000)

[1] The mode of expression is convenient; it must not be taken as implying that these various reactions depend on the presence in the serum of a variety of substances rather than of a substance with a variety of properties.

renders it difficult to escape from the conclusion that there was an infection with paratyphoid B: the similarity of the behaviour of *Valérie* 21 in the patient's serum and in the artificial *Schottmüller B* serum may also be not without significance. Whether a second infection was superadded, and if so with what organism, remain undecided.

Case V. "*Shepherd*" (6. ix. '05). Male, 4 years; clinically atypical typhoid. Two samples of blood gave the following agglutination limits:

	Typhoid *Guy's*	*Brion and Kayser*	*Schott. B*	*Aertryck*	*Gaertner L.I.P.M.*
6. ix. '05	50	0	>500	50	0
14. ix. '05	0	0	10,000	50	0

Repeated attempts were made to isolate paratyphoid bacilli from the urine and faeces by plating and by animal inoculations, but without success. The organism already described under Case IV. (*Valérie* 25) as resembling paratyphoid A was present in large numbers in urine and faeces: it gave no reaction with the patient's serum. The high, and almost pure, agglutination with *Schottmüller B* renders the diagnosis nearly certain.

Summary.

1. Three fully-established, and two probable, cases of infection with *B. paratyphosus B* producing a typhoid-like illness have been found in a series of 176 cases of typhoid.

2. Preliminary culture in a fluid medium containing dulcite is of great assistance in isolating paratyphoid organisms from mixtures such as faeces.

3. The diagnosis of paratyphoid fever can generally be made from the agglutinative reactions of the serum.

4. Absorption tests are necessary for the positive or negative diagnosis of mixed infections.

5. The structure of the agglutinated masses of paratyphoid and Gaertner bacilli varies with homologous and heterologous sera, and is of diagnostic import.

6. Gaertner and paratyphoid agglutinins are more thermostabile than typhoid agglutinin by 5°—10° C.

7. The agglutinations of paratyphoid, hog-cholera and Gaertner bacilli with a series of typhoid sera are described, and the circumstances which influence the presence and amount of these secondary agglutinins discussed.

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THE TOXIN OF THE BACILLUS ENTERITIDIS OF GÄRTNER.

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Cases of illness arising from the ingestion of unsound food may have their origin in food that is really putrid, *i.e.* the material has all the objective appearance of contaminated food, it smells bad, has an unhealthy colour and usually a decidedly peculiar flavour. There are, however, two other forms of unsound food which are much more likely to be dangerous on account of the absence of obvious signs of putridity; in one there is only a very faint odour and the other is quite without smell. The former arises from infection with the *B. botulinus* of van Ermengem, while the latter is caused by the *B. enteritidis* of Gärtner or other allied organisms.

Due attention has been paid to the first two forms of food poisoning, but the same care has not been given to the third—the most dangerous of the three, as cooking does not destroy the toxicity. It was considered, therefore, that an investigation into the toxin of the *B. enteritidis* might be of some value, more especially as most of the previous work done on this bacillus dealt with the organism itself.

Fischer (1902) gives a very excellent résumé to date of the literature of meat poisoning, particularly of the Gärtner or Gärtner-like cases. He states that most of the infected meat comes from animals which have been ill, especially after calving, and which have been killed in order to save them from dying. Some space in this communication is devoted to the consideration of the toxin: the figures given, although somewhat incomplete, yield the main result that one can separate the toxin from a culture of the organism and that the toxin withstands boiling. He also gives a very accurate description of the clinical and post-mortem appearances of the experimental animal.

Bonhoff (1904), working with another member of the same group of organisms, the *B. typhi murium* of Loeffler, likewise obtained a toxic fluid free from bacilli which retained its toxicity after boiling.

Schottmüller (1904) found that he obtained fairly active toxic fluids after boiling cultures of *B. paratyphi*, another closely allied bacillus.

Trautmann (1903), working also with a member of this same group which he had isolated from a case of food poisoning, found that boiled cultures were toxic and that the filtrates from cultures possessed a slight degree of toxicity. According to this author there is some doubt about the heat-resisting properties of the toxin of the *B. morbificans bovis* described by Basenau (1893), and of the Moorseele bacillus of van Ermengem (1903), but the toxin of the bacillus isolated by Kaensche (1896) at Breslau resists boiling.

Van Ermengem (1903) stated that the toxin of the bacillus he found in the Moorseele outbreak did not lose its potency when heated to 100° C. or even to 120° C.

Morgan (1905) obtained a toxin from bacilli of the Gärtner type which he isolated from normal intestines. It was very toxic for guinea-pigs.

Throughout the following experiments mice were used as the test animals as they were found to be particularly susceptible to the toxin of the *B. enteritidis*, and were therefore peculiarly suited for the demonstration of the action of small doses. All injections were intraperitoneal.

The strain of the *B. enteritidis* which was used was, except where otherwise stated, a virulent strain that had been recently repeatedly passed through guinea-pigs. All filtrates were carefully tested as regards their sterility before injection.

1. In the first place an attempt was made to obtain toxic material for the experiments by growing the bacillus in ordinary broth, and, after varying periods of growth, filtering through a Chamberland filter. It was found that cultures up to three days old yielded practically no toxin, although there had been abundant growth in the flasks. The filtrates obtained from the older cultures however showed varying degrees of toxicity. The fact that filtrates from the older cultures were more toxic than those from early growths indicates that the toxin of the Gärtner bacillus is of the class of endotoxins, for it seems probable that it was only after the death of the bacilli and the

subsequent disintegration of the cell bodies that one obtained the toxin in a free state.

TABLE I. *Intraperitoneal injection of Filtrates of broth cultures.*

Age of culture	Amount injected				
	2 c.c.	1 c.c.	0·5 c.c.	0·1 c.c.	0·05 c.c.
3 days	Not dead in 40 days	Dead in 24 hrs.	Not dead in 40 days	Not dead in 40 days	Not dead in 40 days
5 days	Dead in 24 hrs.	Dead in 24 hrs.	Not dead in 40 days	Not dead in 40 days	Not dead in 40 days
7 days	Dead in 5 days	Dead in 10 days	Not dead in 40 days	Not dead in 40 days	Not dead in 40 days
9 days	Dead in 24 hrs.	Not dead in 40 days	Dead in 24 hrs.	Dead in 14 days	Not dead in 40 days

As cultivation in ordinary broth did not yield positive results, other fluid media were tried but without success. Cultures in milk were likewise tried, but these, too, gave quite unsatisfactory results.

2. As it appeared, therefore, impossible to obtain the toxin, at least in reasonable amount, by the above methods, the bacilli themselves were next dealt with. Suspensions of the bacilli in broth heated to 60° C. for 30 minutes proved to be fairly active. Positive results were also obtained with suspensions of bacilli killed by exposure to the vapour of chloroform; these experiments will be referred to in more detail later. It was not, however, until the bacilli were dealt with in mass and material obtained either by autolysis or by grinding in the cold by Macfayden and Rowland's method that one had good and reasonably constant results. The method employed in the present research was the autolytic one.

The manner in which the mass of bacteria for autolysis was obtained and the autolysis carried out was finally as follows: Two to four large (Roux) bottles containing agar were inoculated with an emulsion of the bacilli and after from 18 to 20 hours at 37° C. the resultant growth was brushed off the agar surface with the aid of a sterile brush and a small quantity of salt solution. The emulsion so obtained was then centrifugalised and the bacterial sediment mixed with a little sterile normal saline or distilled water, distributed into sterile bottles in approximately equal amounts, and, after the addition of a drop or two of toluol, placed in an incubator at 37° C. for varying periods. At the end of the period allowed for autolysis, the mass was diluted with an equal volume of distilled water or normal saline and filtered through a Chamberland filter.

As regards the time requisite for autolysis, it was found that about eight or nine days was on the whole the best. Before the expiration of that time one frequently, it is true, obtained toxic filtrates but they were not quite reliable. On the other hand if the autolysis was allowed to go on too long one obtained filtrates which were quite innocuous.

TABLE II. *Autolysis without toluol.*

Duration of autolysis	Filtrate. Amount injected					Unfiltered emulsion. Amount injected			
	2 c.c.	1 c.c.	0·5 c.c.	0·2 c.c.	0·1 c.c.	1 c.c.	0·5 c.c.	0·2 c.c.	0·1 c.c.
5 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	—	Not dead in 40 days	—	—	—	—
9 days	—	Dead in 2 days	Dead in 35 days	Dead in 11 days	Dead in 35 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 2 days	Dead in 30 days
19 days	—	All alive 40 days after injection				Dead in 24 hrs.	Dead in 24 hrs.	Dead in 2 days	Not dead in 40 days

It is to be noted, however, that toluol, which was added in the first instance to prevent growth of any foreign bacteria which had possibly obtained entrance to the mass, apparently rendered the autolysis more rapid. In this series, as in that without toluol, too prolonged digestion caused the disappearance of the toxin from the filtrate.

TABLE III. *Autolysis with toluol.*

Duration of autolysis	Filtrate. Amount injected				Unfiltered emulsion. Amount injected			
	2 c.c.	1 c.c.	0·5 c.c.	0·2 c.c.	1 c.c.	0·5 c.c.	0·2 c.c.	0·05 c.c.
6 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Not dead in 40 days
	1 c.c.	0·5 c.c.	0·2 c.c.	0·1 c.c.	1 c.c.	0·5 c.c.	0·2 c.c.	0·1 c.c.
10 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 5 days	Not dead in 40 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 2 days
19 days	Not dead in 40 days	Not dead in 40 days	Dead in 31 days	Not dead in 40 days	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 2 days

In both sets of experiments the toxicity of the autolysed mass was tested as well as the toxicity of the filtrates, the fluid or emulsion being rendered absolutely sterile by heating for 30 minutes at 60° C. In every case this preparation was much more toxic than the filtrate. It will be noted that although the filtrates after prolonged autolysis were atoxic, such was not the case with the bacterial mass. The explanation of this fact may be that it is only the free toxin which is destroyed, whereas that which still remains within the bodies of the bacilli and which would naturally be effective on injection is untouched. Some

support is lent to this view by the fact that cell-juice obtained by cold grinding very rapidly deteriorates even when kept in the cold.

A few experiments were carried out to see whether one could not obtain a still more toxic filtrate in the autolytic experiments, if, instead of leaving the bottles containing the autolysing bacteria at rest, they were repeatedly shaken. The result seems rather to point to the fact that the shaking on the whole gives more toxic filtrates.

TABLE IV. *Filtrate from* 8 *days' autolysis of Gärtner's bacillus.*

	Shaken	Unshaken
Dose in c.c.	Effect	Effect
1·0	Dead in 2 days	Dead in 5 days
0·5	Dead in 2 days	Not dead in 30 days
0·2	Dead in 2 days	Not dead in 30 days
0·1	Not dead in 30 days	Not dead in 30 days
Filtrate from 10 *days' autolysis of Paratyphoid B.*		
1·0	Dead in 24 hrs.	Dead in 24 hrs.
0·5	Dead in 24 hrs.	Dead in 2 days
0·2	Dead in 2 days	Dead in 2 days
0·1	Dead in 24 hrs.	Dead in 12 days

3. The question as to what part the medium in which the autolysis was carried out played was next considered and several experiments under this head were done. For instance the distilled water or normal saline was replaced by a 1 % sodium carbonate solution or by a 2 % cane sugar solution, but it was found that they either hindered autolysis or destroyed the toxin as soon as formed. With sugar the autolysis on inspection appeared to be far from complete, whereas with the sodium carbonate it seemed to have progressed further than usual.

TABLE V. *Autolysis of Gärtner bacilli in varying media.*
(9 *days autolysed.*)

	Dose of Filtrate	
Medium	0·2 c.c.	0·1 c.c.
Aq. dist.	Died in 6 days	Died in 6 days
Sod. Carb. sol. 1 %	Not dead in 40 days	Not dead in 40 days
Cane Sugar sol. 2 %	Ditto	Ditto

The influence of the use of chloroform vapour in the preparation of the *toxic* material was considerable. Ordinary test-tube cultures 18 to 20 hours old had the wool plugs removed and corks substituted so

as to hold small pieces of cotton wool saturated with chloroform in position. The tubes were then placed in the incubator for two to three hours on their sides with the agar slightly elevated to prevent any of the chloroform running down on to the growth. In every case the bacteria on testing were found to be dead. Suspensions made from bacilli so prepared were found to be much less toxic than suspensions made with the same quantity of organisms suspended in the same amount of broth and made from cultures of exactly the same age, but sterilised by heating to 60° C. for 30 minutes.

TABLE VI. *Suspension of 4 mgm. Gärtner culture in 2 c.c. broth. Cultures killed by $CHCl_3$ vapour and by heat.*

Dose in c.c.	$CHCl_3$	Heat (30 mins., 60°)
1·0	Dead in 5 days	Dead in 4 days
0·5	Not dead in 30 days	Dead in 2 days
0·2	Not dead in 30 days	Dead in 2 days
0·1	Not dead in 30 days	Not dead in 30 days

4. As regards the effect of heat it was proved conclusively that the statement that this toxin can stand boiling without being destroyed is correct. Very many experiments were made on this point and all gave similar results.

TABLE VII. *Autolysed Gärtner bacilli heated for 30 mins. to different temperatures. Dose injected 0·5 c.c.*

Temp.	Result
60°	Dead in 2 days
80°	Ditto
90°	Ditto
100°	Ditto

TABLE VIII. *Autolysed Gärtner bacilli heated to 60° for 30 mins.*

Dose in c.c.	Autolysed 9 days	Autolysed 8 days
1·0	Dead in 24 hrs.	Dead in 24 hrs.
0·5	Ditto	Dead in 48 hrs.
0·2	Ditto	Dead in 24 hrs.
0·1	Ditto	Dead in 48 hrs.

A like result was obtained with guinea-pigs instead of mice and also when some pure cell-juice of the *B. enteritidis*, kindly given me by Dr Macfadyen, was used.

TABLE IX. *Gärtner bacilli autolysed* 10 *days, heated for* 30 *mins., dose* 1 *c.c. injected intraperitoneally into guinea-pigs of about* 150 *grms.*

Heated at 100°	Dead in 24 hrs.
,, 90°	Ditto
,, 80°	Lived
,, 70°	Dead in 24 hrs.
,, 60°	Ditto

TABLE X. *Gärtner cell-juice heated for* 30 *mins., dose* 0·1 *c.c. injected intraperitoneally into guinea-pigs of about* 350 *grms.*

Control (unheated juice)	Dead in 20 days
Heated at 100°	Dead in 21 days
,, 80°	Dead in 1 day
,, 70°	Dead in 21 days
,, 60°	Dead in 2 days

5. The question as to whether in the case of the *B. enteritidis* virulence and toxicity go hand in hand or are independent of one another came under review, but, as has proved to be the case with other organisms, these two properties were shown not to be in complete correspondence. The two strains of *B. enteritidis* used showed very well-marked differences in virulence; one had been isolated years ago and the other was the standard virulent culture used throughout these experiments and passed through guinea-pigs twice just before this experiment.

TABLE XI. *Virulence—tested on guinea-pigs.*

Old strain M.L.D. $= \frac{1}{10}$ of a loop.

Virulent strain M.L.D. $= \frac{1}{500}$ of a loop.

1 loop = 2 mg. moist culture.

The results obtained show a somewhat increased toxicity in the case of the virulent organism both when the toxic fluid from autolysis was used, and, under approximately quantitative conditions, when suspensions of equal amounts of bacilli killed by means of heat were taken.

TABLE XII. *Autolysed bacilli diluted with* 3 *volumes of dist. water heated to* 60° *for* 40 *mins.*

	Nature of culture. (Gärtner)	
Dose in c.c.	"Old"	"Virulent"
1·0	Dead in 24 hrs.	Dead in 24 hrs.
0·5	Dead in 24 hrs.	Dead in 24 hrs.
0·2	Not dead in 15 days	Dead in 24 hrs.
0·1	Not dead in 15 days	Dead in 24 hrs.

Suspensions of 2 *mg. cult. in* 2 *c.c. broth, heated to* 100° *for* 30 *mins.*

Nature of culture	Dose injected	
	0·5 c.c.	0·2 c.c.
Gärtner "Old"	Dead in 24 hrs.	Not dead in 6 days
,, "Virulent"	Dead in 24 hrs.	Dead in 2 days

6. A few experiments were carried out with the colon bacillus and the paratyphoid bacillus as regards their production of an endotoxin and in both cases results very similar to those with Gärtner were obtained. In the case of the paratyphoid B. bacillus the toxin was found to

TABLE XIII. *Paratyphoid B. bacilli autolysed for* 9 *days.*

Dose in c.c.	Filtrate	Unfiltered emulsion	
		Unheated	Heated to 60° for 30 mins.
1·0	Dead in 24 hrs.	Dead in 24 hrs.	Dead in 24 hrs.
0·5	Dead in 24 hrs.	Ditto	Ditto
0·2	Dead in 2 days	Ditto	Ditto
0·1	Dead in 24 hrs.	Ditto	Ditto

withstand heating to 100° as was noted by Schottmüller (*l.c.*), but 30 mins. at 100° rendered a suspension of fairly virulent colon bacilli innocuous. Schwarz (1906) has, however, recently noted that filtered

TABLE XIV. *Paratyphoid B. bacilli autolysed* 11 *days, heated for* 30 *mins., dose* 0·5 *c.c.*

Heated at 100° C.	Dead in 2 days
,, 90°	Dead in 1 day
,, 80°	Ditto
,, 70°	Ditto
,, 60°	Ditto

cultures of a variety of *B. coli* isolated from a spontaneous disease in a guinea-pig were very toxic to mice, guinea-pigs and rabbits after boiling. The clinical and post-mortem signs to which reference will presently be made were however somewhat different.

7. Feeding animals with infected meat was also tried, the meat being used both cooked and uncooked. As in the experiments of Fischer (*l.c.*), Trautmann (*l.c.*) and others, positive results were obtained. In the present instance meat for feeding purposes was obtained by killing a guinea-pig with a dose of living virulent culture. From the cadaver of the guinea-pig, muscle, spleen, liver, etc., were removed; in one case it was given to mice as it was, and in the other the material was mixed with a small quantity of water and then the whole was boiled for about half-an-hour. The meat, strained off from the broth, was given

to mice and the broth taken up with bread was used to feed still other animals. Death was delayed, in some cases, for quite a long period. As will be seen from the table, both animals fed with raw meat died, both with the cooked meat, and only one from the broth test. Mice from the same batch of animals, fed on ordinary diet, remained quite healthy throughout this period.

TABLE XV.

Nature of the food		Result
Raw	(*a*)	Dead in 8 days
	(*b*)	,, 15 ,,
Cooked	(*a*)	Dead in 30 days
	(*b*)	,, 38 ,,
Broth	(*a*)	Dead in 9 days
	(*b*)	Not dead in 50 days

P.M. signs were on the whole quite characteristic.
Gärtner bacilli recovered from heart's blood in raw feeding.

8. The clinical symptoms arising from poisoning with the Gärtner toxin are very marked and characteristic. At varying periods after the injection the experimental animal becomes comatose, and lies on its side with scarcely a movement. The eyelids are glued together. If, however, one pinches its tail or applies some other strong stimulus, the animal gives a few convulsive kicks with its four legs but quickly relapses into the comatose state. This state has been observed to last for as long as nine hours before death. There appears also to be some diarrhoea.

A post-mortem examination was always made, and the sterility of the fluids injected was always controlled by means of cultures made from the peritoneal fluid and often also from the heart's blood. In many cases organisms were obtained other than the Gärtner bacillus. A notable sign with both Gärtner and paratyphoid was the rapidity of the onset of putrefaction. The post-mortem appearances had much in common with those arising from other intestinal intoxications, *i.e.* hyperaemia of lung, spleen, suprarenals, etc. In Gärtner poisoning the filling of the small intestine with a clear yellow fluid was practically a constant phenomenon although the same was noted in some of the deaths from *coli* and paratyphoid B. infection. Another lesion which was noted fairly frequently in Gärtner animals and in one at least of the paratyphoid mice was small necrotic areas in the liver; these were also observed previously by Trautmann. Ballard (1890–1891) gives a very similar post-mortem picture for fatal cases in man.

The clinical appearances of paratyphoid B. intoxication so far as could be observed were very like those described for Gärtner poisoning,

but in the case of *coli* infection in the present experiments neither the narcosis nor the gluing together of the eyelids was noted.

In conclusion I wish to offer my best thanks to Dr Macfadyen for much kindly advice and criticism during the course of the above research.

SUMMARY OF RESULTS.

1. Cultures of Gärtner's bacillus grown on broth do not excrete any large amount of toxin into the fluid medium.

2. Suspension in broth made from ordinary agar cultures and killed by means of heat (30 mins. at 60° C.) are fairly toxic.

3. The most toxic preparations were obtained by autolysis of the bacilli, especially in the presence of toluol.

4. Cultures grown on agar and killed by means of chloroform vapour lose their toxicity to a considerable extent.

5. Autolysis takes place best in the presence of distilled water or of normal saline.

6. Autolysed material sterilised by heat is more toxic than the filtrate obtained from the same digest.

7. Shaking the bottles during the process of autolysis increases the yield of toxic substances.

8. Gärtner toxin withstands heating to 100° C. for 30 mins.

9. Paratyphoid B. bacillus cultures on autolysis yield a filtrate quite as toxic as those from the Gärtner bacillus itself.

10. Paratyphoid toxin is also heat resistant (100° for 30 mins.).

11. A colon bacillus gave a toxin which was fatal to mice. It was not heat resistant.

12. Feeding experiments with Gärtner-infected meat, both cooked and uncooked, proved successful.

13. So far as the present experiments go the connection between virulence and toxicity does not appear to be very definite.

14. The Gärtner bacillus contains a toxin of the endotoxin type as is shown in comparing results 1 and 3. This toxin gives rise to very definite clinical symptoms of which the gluing together of the eyelids and the prolonged narcosis before death are the most notable. The post-mortem signs have much in common with those arising from other intestinal intoxications.

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UPON THE REDUCTION OF METHYLENE BLUE BY COW'S MILK.

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H. Smidt in a recent note on the reduction of methylene blue by means of milk[1] confirmed the results of Schardinger that a mixture of formaldehyde and methylene blue is decolorised by the action of milk. This reaction depends on the presence of a ferment—a catalase—in the milk, and, as this ferment is readily destroyed by heat, serves as a very simple method of determining whether a given sample of milk is "raw" or not. By way of conclusion Smidt stated that he thought this test might be of still further utility in that it might be possible to estimate the degree of bacterial contamination of the milk by its means. If such a comparatively simple test could be employed, in an easy and effective fashion, it would be of immense service, as, owing to the rapidity with which it can be carried out, the milk could be examined before distribution.

As the possibilities of this test seemed to be large I carried out a few experiments. In the first place it was of some importance to know whether the time required for reduction was independent of temperature, within reasonable limits.

The experiments were performed in a water-bath constant to within 1 degree when used with care, made of glass, so that the test-tubes, containing the milk and methylene blue, could be observed without being disturbed. The quantity of milk used was 10 c.c., pipetted into test-tubes of as near equal bore as could be obtained, and to this milk was added 0·5 c.c. of Smidt's methylene blue solution[2]. The blue and the

[1] *Hygien. Rundsch.* No. 23, 1903.

[2] Methylene blue solution. Saturated meth. blue alcoholic (absolute) sol. 5 c.c.
Formaldehyde sol. 5 c.c.
Aq. dist. 190 c.c.

milk were mixed by shaking and then placed in a circular test-tube rack which was immersed in the water-bath. It was found that the variation in time required for reduction, between the temperature of 40 and 50 degrees was very considerable. This demonstrates in the first place the fact that, if the temperature were not very closely controlled, great mistakes as to the reducing power of the milk, and so as to its bacterial content, might easily be made. A rise of 10° caused a diminution of practically 50 per cent. in the time of reduction.

TABLE I.

Milk (fresh), 10 c.c. 0·5 meth. blue sol.

Temp. of bath	Time required for reduction
40°	(a) 15 mins. (b) 15 mins. 30 secs.
45°	(a) 10 mins. 40 secs. (b) 11 mins.
50°	(a) 7 mins. 45 secs. (b) 8 mins.

Thus it will be seen that to get anything like reliable and constant figures a very definite temperature must be maintained.

Another point which was of interest was to see whether the temperature of the milk itself played any part. This was important as in some instances the milk might be brought for examination in an ice box and a sample taken for immediate testing. Here again another possible source of error was detected. It must be admitted, however, that the difference was not very marked.

TABLE II.

Water-bath at 50°.

(*a*) 10 c.c. milk at room temp. (18° C.) had 0·5 c.c. meth. blue added and then placed in the bath. Time for reduct. 9 mins. 30 secs.

(*b*) 10 c.c. of same milk, but previously in bath at 50° for 7 mins., had 0·5 c.c. meth. blue added, then returned to bath. Time for reduct. 8 mins. 45 secs.

As the catalase is found almost completely in the cream, as is readily proved by centrifugalising the milk, it was interesting to see if allowing the milk to stand for a few hours and then taking two samples, one from the top layer the other from the bottom, would alter the times obtained. Milk was accordingly left absolutely still for some three hours and a sample taken from the top layer by means of a pipette and another from the bottom of the vessel. Here again a result was obtained in favour of the multiplication of errors.

TABLE III.

(*a*) Milk from bottom reduced in 8 mins. 45 secs.
(*b*) ,, top ,, ,, 6 mins. 30 secs.

Some experiments were also carried out as regards the effect of previously heating the milk. The results showed that the catalase is very sensitive to the action of heat. According to my results the point of destruction lies somewhere between 65 and 70 degrees. This figure is a few degrees lower than that given by Smidt.

TABLE IV.

Milk and meth. blue as usual. Bath 50°.

Temp.	Time of exposure	Time required for reduction
60°	10 mins.	10 mins. 45 secs.
	20 mins.	12 mins. 50 secs.
	30 mins.	13 mins. 15 secs.
65°	10 mins.	8 mins. 15 secs.
	20 mins.	16 mins.
	30 mins.	Slight in 30 mins.
70°	10 mins.	30 mins. no reduction.
	20 mins.	,, ,, ,,
	30 mins.	,, ,, ,,

Control (unheated milk) 7 mins. 50 secs.

Other experiments, roughly quantitative in nature, were performed in order to see whether or not the catalase had been injuriously affected by heating at 60°. Here again the milk was exposed for different periods at the given temperature. In this case known volumes of the methylene blue solution were added until the milk ceased to decolorise it. As will be seen from the following table, the catalase had been affected most by the 30 minutes exposure. There is even a slight difference between that exposed for 10 minutes and the control. In this experiment the milk which was used was one day old but had been kept in the ice box overnight.

TABLE V.

Amount of meth. blue	Milk control	Milk heated to 60° for 10 mins.	20 mins.	30 mins.
0·5 c.c.	2 mins. 15 secs.	2 mins. 45 secs.	3 mins. 0 secs.	3 mins. 20 secs.
+0·5 c.c.	3 ,, 25 ,,	2 ,, 30 ,,	3 ,, 30 ,,	6 ,, 10 ,,
+0·5 c.c.	5 ,, 0 ,,	6 ,, 0 ,,	9 ,, 5 ,,	Slight reduct. in 30 mins.
+0·5 c.c.	Slight reduct. in 30 mins.	No reduct. in 30 mins.	No reduct. in 30 mins.	

This experiment further serves to demonstrate the fact that old milk reduces methylene blue more rapidly than fresh. This of course is presumably due to the increased bacterial content.

As so many factors, which of themselves might not be looked on as particularly weighty, seem to affect the easy carrying out of this test it was considered of importance to see what the result of adding varying amounts of an organism like *B. coli* might be. As will be seen from the table it was only on adoption of the quantitative method that one got any result, and at the best the figures obtained are not very striking.

TABLE VI.

Milk 10 c.c. + meth. blue + broth cult. of *B. coli*. Bath at 50°.

Amount of meth. blue	Amount of *B. coli* cult. 0·2 c.c.	0·5 c.c.	1 c.c.
0·5 c.c.	9 mins. 50 secs.	9 mins. 45 secs.	9 mins. 30 secs.
0·5 c.c.	1 min. 45 secs.	1 min. 45 secs.	1 min. 25 secs.
1·0 c.c.	No reduct. in 30 mins.	Slight reduct. in 30 mins.	About half reduced in 30 mins.

Another source of error which I should imagine would prove fatal to the quantitative use of this test as a means of finding the bacterial contamination of milk is that the reducing action of organisms varies to a marked degree. In a paper published by Prof. Hahn and myself[1] we showed this variation very clearly. The following table, in order to demonstrate this point, is extracted from the paper in question.

TABLE VII.

Reduction of 0·5 c.c. 1/10 % meth. blue sol. by 10 c.c. bouillon culture.

Culture and age	Reduction time Mins.	Secs.
Bac. coli (18 hours)	5	10
Bac. typhi (18 hours)	20	—
Bac. prodigiosus (2 days)	18	30
Staphylococcus aureus (2 days)	6	—
Bac. lact. aerogenes (2 days)	7	—
Bac. subtilis (2 days)	Only slight reduct. in 1 hour.	
Bac. diphtheriae (2 days)	8	—

Conclusions:—The methylene blue formaline test serves most excellently to differentiate between boiled (or heated) and "raw" fresh milk. As a means of estimating the bacterial contamination of the milk it seems to me to be too delicate for ordinary rough and ready use. It might possibly be of value in a large laboratory where the various sources of error could be readily eliminated.

[1] *Arch. f. Hygiene*, vol. XLIV. p. 296.

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THE BACTERIAL FLORA OF "BLOWN" TINS OF PRESERVED FOOD.

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So far as I am aware the literature on the nature of the causes to which gaseous decomposition of tinned food is due is extremely scanty. Pfuhl and his collaborators have, it is true, published one or two papers on this subject, but their attention has in the main been devoted to the practical side of the preparation of tinned food. In one paper[1] Pfuhl mentions that out of 106 tins of preserved food, 29 contained organisms but does not state their nature. In a still more recent publication[2] Pfuhl and Wintgen refer to a non-bacterial cause of gaseous decomposition in tinned foods, viz. the formation of hydrogen from chemical reactions going on between the food and the metal of the box. The following short note on the bacterial flora accordingly may be of some interest.

Through the kindness of Dr Alexander, Medical Officer of Health for Poplar, I was enabled to obtain a variety of "blown" tins which had been condemned as unfit for food by his inspectors.

In the present note I intend dealing most fully with the organisms obtained from the decomposed contents of several boxes of sardines. These boxes had all the typical signs of "blown" tins, bulging sides, etc. On opening them the gas which escaped was extremely foetid in odour. The flesh of the sardines however looked quite normal and healthy.

Cultures from the contents of the tins were in each case made in two different fashions, (1) a small portion of the sardine was removed by means of a sterile platinum loop and then emulsified with sterile normal saline. Lactose neutral red agar plates were inoculated direct by means of a bent glass rod from this emulsion, and grown both aerobically and anaerobically. (2) A tube of broth, inoculated with a small portion of the sardine, was cultivated at 37° for some 20 hours and then plates similar to the above prepared and grown in like manner. Anaerobic cultivation was carried out in the Bulloch apparatus, in an

[1] *Zeitschr. f. Hygiene*, 1904, Bd. 48. [2] *Ibid.* 1905, Bd. 52.

atmosphere of hydrogen. The tins were, it may be here remarked, opened carefully with a sterilised instrument so as to avoid as far as possible any contamination from outside.

At the end of the first 24 hours there was some growth in both sets of plates, both aerobic and anaerobic; and by the end of 48 hours growth was good in all. Subcultures on the various sugar media, dextrose, lactose, cane-sugar, dulcite, mannite, and on milk were made from the different colonies on the various plates. These were all grown aerobically, as the organisms from the anaerobic plates were found after examination to be the same as the aerobic growths. The results obtained after three days' growth showed that four different kinds of organisms had been isolated from the tin whose contents were being tested.

	I	III	VI	VIII
Dextrose	acid and gas	acid and gas	acid	acid
Lactose	–	acid and gas	–	–
Cane-sugar	–	–	–	–
Dulcite	–	acid and gas	–	–
Mannite	acid	acid and gas	acid	acid and gas
Milk	–	acid	acid	very acid
Milk clot	–	+	–	partial +
Indol (6 days)	+	+	+ +	+ +

On further examination of these four cultures it was found that that numbered III was a colon bacillus, thus leaving three other unknown but apparently allied cultures.

To settle the question as to whether these cultures were pathogenic or not, injections were made intraperitoneally into guinea-pigs, with the result that after doses of 1 c.c. of 20 hours' broth cultures the animal which had been inoculated with culture I died the following day, that with culture VIII ten days after, whilst that with culture VI was quite well and healthy forty days later. In both of the fatal cases the organisms which had been injected were recovered from the peritoneal fluid.

It was further of some interest to see whether inoculation of boxes of fresh uncontaminated sardines with these various cultures would give rise to gaseous decomposition. On July 10 four tins of sardines were therefore inoculated with cultures I, VI, VIII, and *B. coli*, another tin obtained at the same time as the others being kept untouched as a control. On Sept. 25 the tins, which had been kept during the intervening period at room temperature, were examined. It was noted that the tins which had been inoculated with culture I and *B. coli* both felt blown and had the typical appearance associated with that condition

and further on opening the tins, from the two just mentioned there was a slight escape of gas, but no foetid odour. Cultures were made from all the tins; and abundant growth, except in the case of the control, which was sterile, of the organisms with which they were injected was obtained. Culture I now gave a small quantity of gas when grown on mannite broth.

From other experiments, using material obtained from beef, salmon, and sardine tins, the results were practically always the same, *i.e.* one obtained cultures of organisms which all conformed more or less to the intestinal type. For instance, as is seen from the following short table, two varieties of organisms were obtained from a "blown" beef tin and two from a "blown" salmon tin.

	Beef		Salmon	
	1	2	1	2
Dextrose	acid and gas	no effect on	acid and gas	acid and gas
Lactose	acid and gas	any sugar	–	–
Cane-sugar	acid and gas		acid and gas	–
Dulcite	–		–	–
Mannite	acid and gas		acid and gas	–
Milk	acid (no clot)		acid (no clot)	slight acid then alkaline

I may add that no ill result occurred either on feeding animals with the decomposed beef or salmon or on injecting a watery extract of the same material (2 c.c.) intraperitoneally into guinea-pigs.

From still another "blown" box of sardines some varieties of organisms were isolated:

	a	*b*	*c*	*d*	*e*	*f*	*g*
Dextrose	acid and gas	acid	acid	acid and gas	acid and gas	acid	acid
Lactose	acid and gas	acid	acid	acid	acid	–	acid
Cane-sugar	acid	acid	–	acid	acid	acid	acid
Dulcite	–	–	–	–	–	–	–
Mannite	acid and gas	acid	–	acid and gas	acid and gas	acid	acid
Milk	acid	acid	acid	acid	alkaline	acid	acid
Milk clot	+	+	+	+	–	+	–
Indol (6 days)	trace	trace	trace	slight	distinct	slight	distinct

Summary.

In the samples of "blown" preserve tins examined by me, organisms of an intestinal type were present, which on reinoculation into sound tins gave rise to a gaseous decomposition.

No toxic symptoms were produced on feeding guinea-pigs with the contents of the "blown" tins.

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ON THE ABSORPTION OF PROTEIDS FROM THE INTESTINE.

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Much of the recent work upon the enzymes which are secreted into the alimentary tract has made it probable that in the digestion of proteids hydrolysis is carried further than used commonly to be supposed. Kühne[1] 40 years ago first showed that proteids were partially destroyed by trypsine, and that when fibrin was introduced into the duodenum leucine and tyrosine were formed by the action of trypsine, in appreciable quantities, in the intestine itself no less than *in vitro*; but he thought that proteid which was so completely hydrolysed as this was wasted, and therefore that it was improbable that such complete hydrolysis took place to any extent in normal digestion. He also thought that one-half of the proteid molecule resisted the action of this enzyme entirely, and retained its proteid character when the enzyme had done its utmost. Kutscher[2] and others have since shown that tryptic action may completely break up everything that gives the biuret reaction, Zunz[3] and others that pepsine also destroys proteid, and Cohnheim[4] that another ferment exists in the intestinal mucous membrane, and also in the secretion of the small intestine, which hydrolyses peptones and albumoses and abolishes the biuret reaction in solutions of these substances. It seems therefore possible that the reason why the uncoagulable proteids formed in digestion are not found in the blood during absorption of proteids is, not that they are synthetized into coagulable proteids during their transit through the intestinal mucous membrane, but that they are still further hydrolysed and converted into substances which cannot be detected in the blood by the biuret reaction because they do not give this reaction, and in fact are the amido-acids simple and compound which are the final products of proteid hydrolysis.

In order to put this conception to the test some method must be devised for detecting such substances in the blood during the absorption of proteids. Kutscher and Seemann[5] tried to identify individual

amido-acids like leucine and tyrosine, which owing to their insolubility and the readiness with which they crystallise might be expected to separate out on evaporating down the filtrate obtained after coagulating the blood by heat. As is well known they failed to detect any such crystalline derivatives of proteids in the blood removed from dogs during the digestion of meat. Accordingly they concluded, as others had done before, that proteid food after it has been hydrolysed in digestion undergoes synthetic changes in the intestinal mucous membrane before it is handed on for distribution to the blood.

It is a question whether the failure to detect crystalline derivatives under these conditions in the blood proves that such substances were not absorbed into the blood. It is true that large quantities of proteid had been given to the animals in their experiments, and that probably the greater part of this had been absorbed. But it does not follow that all that had reached the blood was still in the blood. The dogs were given two meals, each of about 100 grammes of proteid, 20 hours and 12 hours respectively before the blood was collected for examination. How much of the nitrogen contained in these meals had been absorbed was not determined; it is merely stated that the stomach and intestines in each case were found to be moderately full. Neither do we know how much of the absorbed nitrogen had been excreted by the kidneys. And if all that was absorbed reached the blood in the form of amido-acids and similar substances, there may well have been only traces still circulating in the blood when it was collected twenty hours after absorption began. We do not know at what rate these substances are removed from the blood by the tissue cells which feed on what the blood brings to them. It is certain that the amount of blood that passed through the intestine in the course of the 20 hours after the first meal was enormous compared with the volume that could be collected from the portal vein when the animal was bled. So that even if absorption was still going on while the blood was being collected it is possible that only a very minute fraction of the absorbed substances was present in the blood that was examined. And there is a limit to the amount of even leucine and tyrosine which can be detected by crystallization from blood after coagulation, a limit which leaves it an open question whether all the proteid that was absorbed did not after all gain the blood in the completely hydrolysed condition.

von Bergmann and Langstein[6] have more recently examined what they call the residual nitrogen of the serum, the nitrogen left in solution after removal of substances coagulable by heat, and they find

that this residual nitrogen in dogs during digestion may amount to 14·77 of the total nitrogen of the serum. They think there is some relation between the amount of residual nitrogen and the absorption of proteids. But they carried out only a few experiments, and on this point these are not conclusive, and they did not compare the composition of the blood in the same animal before and after digestion.

It seemed to us that this point was of sufficient importance to merit special investigation.

The experiments carried out by Salvioli[7] in Ludwig's laboratory in 1880 have often been quoted in support of the theory that the peptones absorbed in the bowel are converted by the cells of the mucous membrane into coagulable proteids. In these experiments a small piece of bowel from 4 to 6 inches long was excised, cannulæ put into the artery and vein supplying it, and blood circulated through them under a constant pressure of 60 mm. Hg. for rabbit's, and 75 mm. for dog's intestine, while the temperature of blood and intestine was maintained at 37° C. The blood was a mixture of 30 parts of calf's blood and 70 parts of a 0·75 % sodium chloride solution. The intestine exhibited active movements for some three or four hours. At the end of that time it was found that the bowel was visibly fuller than before, and in some cases it was noted that blood and a grumous material was found in it. At the beginning of the perfusion 20 c.c. of a 10 % solution of peptone was introduced into the bowel: and at the end of the time it was found that the fluid contained about half a gramme of coagulable proteid, but no peptone. The blood that had circulated as is well known was also found to contain no peptone.

Our first experiments were planned on the same lines. But we used a much greater length of bowel and tied our cannulæ into the main trunk of the superior mesenteric artery and vein: and for perfusion we used the apparatus devised by Martin in which the blood is oxygenated by being pumped through the lungs excised from a defibrinated dog. The blood used was that of the dog that supplied the lungs. Solutions of peptone or of the products of pancreatic digestion were introduced into the bowel in small quantities at a time. Like Salvioli we found that the movements of the intestine were active, in fact abnormally active, and as in his experiments the intestine became distended after an hour or an hour and a half; and when peptone had been put into it a considerable amount of the peptone was missing when the experiment was stopped. The contents of the bowel were collected and measured and the whole length of bowel washed out with normal

the total nitrogen in the solids of the liver, while in one case it was less, and in the other more than this. Whether this means anything more than that the analytical methods employed were defective we are not prepared to say. Circumstances have prevented us from investigating this point further.

So far as the analyses of the liver go therefore they point to this organ as being to some extent a place in which simple nitrogenous derivatives of proteids are deposited during the absorption of digested proteid material. Roughly about the same amount of these substances was detected in the liver as in the blood, and altogether therefore, about 30 % of the absorbed nitrogen may be traced in these two situations.

Putting the results described together it is clear that they are not decisive against the theory of intestinal synthesis of proteids. They are compatible with it, inasmuch as we are not able to trace the whole of the nitrogen that had been absorbed. In Exp. 8 for instance the absorbed nitrogen was equal to 432 c.c. $\frac{N}{10}$ NH_3: of this in the blood we accounted only for 46 c.c., in the liver for 65 c.c., and only if we may take the urinary nitrogen into account can we get anywhere near the total amount. In that case we should have traced altogether 307 c.c. out of 432 c.c. Generally we found a little more in the blood, and in one case more than twice as much.

But according to the theory that digested proteids are synthesized in the intestinal mucous membrane into coagulable proteids, such as are found in the blood, it is conceivable that an increase in the coagulable proteid might be detected in the blood. We can only say that we could not detect it. The concentration of the blood was always altered, but the ratio of nitrogen in combinations precipitated by tannic acid to that in forms that escape precipitation was always diminished, not increased. And the change in concentration of the blood as determined by the hæmoglobinometric method was not different from the change as determined by the total nitrogen. If the absorbed digestion products reached the blood as coagulable proteid and stayed in the blood in this form then the change in concentration of the blood due to absorbed water should appear greater colorimetrically than by the estimation of the total nitrogen: but this is not the case. The agreement between the two methods of determining the dilution of the blood was generally close; taking the mean of all determinations it came out to 11·66 % dilution colorimetrically and 12·33 % by the total nitrogen estimation.

We lay no stress on the fact that it is this latter figure which is the higher of the two. We do not think that the question is capable of being decided in this way. We incline to look on the blood not as a storage place for nitrogenous nutriment but as concerned simply with the transport of such material. The nitrogenous substances removed by the blood from the intestine are we believe fixed in the tissues almost as rapidly as they are absorbed, and that is why they are not to be found in larger quantities in the blood. And also, it must be remembered, there are many grounds for thinking that much of the absorbed products of proteid digestion lose their nitrogen in the process of absorption ; the larger amount of ammonia in portal than in systemic blood during digestion, the extremely rapid excretion of urea after a proteid meal, which in dogs has been shown to result in the excretion of the whole nitrogen of the meal within an hour or two of its absorption, the denitrification processes described by Jacoby[10], Lang and others as part of the surviving metabolism of most organs of the body, but especially the intestine and liver, these as well as the observations of Folin[11] and others on the distribution of urinary nitrogen among the different components of the urine under different conditions, all point to the storage of proteid food in non-nitrogenous combinations and not as resynthesised coagulable proteids.

We therefore are inclined to account for the small fraction, 15 % on an average, of the nitrogen absorbed which we were able to detect in the tannic acid filtrates of the blood, not by supposing that the bulk is resynthesised in the intestine and converted into coagulable proteid, and so lost among the other coagulable proteids of the blood, but partly by supposing that a considerable part of the nitrogenous substances lose their nitrogen in process of absorption in the intestine, and that the rest on reaching the blood are rapidly removed by the various tissues not only by the liver, but by the liver as well as the other tissues, and then in these tissues the denitrification processes are continued. The amount of amido-acids and similar compounds found at any moment in the blood should not be taken, we contend, as an index of the amount of the absorbed nitrogen which has been absorbed in this form, any more than Kühne, Schmidt-Mülheim and others were right in arguing from the fact that the amount of amido-acids to be found in the intestine was small that only small quantities of these bodies are formed in digestion. If formed they are formed for absorption, not for accumulation in the intestine. So, too, with the absorbed amido-acids, they are not absorbed to be stored in the blood, they are removed from

the blood by the cells of the tissues and stored or dealt with there, so that the amount found at any moment still circulating in the blood is only a fraction of that which has been absorbed.

Conclusions.

1. Salvioli's experiments do not prove that peptone which is absorbed into the intestinal mucous membrane is there converted into albumin and globulin. He did not prove that any nitrogenous substance was absorbed, and under similar conditions we could not obtain evidence of any absorption at all.

2. When "peptone," albumoses, or the final products of pancreatic digestion, are absorbed from the intestine there is a constant small increase in the amount of nitrogenous substances contained in the blood which are not precipitated by tannic acid: this is sufficient to account for about 15 % of the nitrogen absorbed.

3. There is a similar increase in these substances in the liver.

4. A flow of urine may be set up by the absorption of such substance in dogs previously in a condition of inanition and under anæsthetics, and in this urine a very considerable amount of nitrogen may appear.

REFERENCES.

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(3) Zunz. *Ibid*, xxiii. p. 146. 1899. et Lavroff. *Ibid*, xxvi. p. 513. 1898; xxxiii. p. 312. 1901.

(4) Cohnheim. *Ibid*, xxxv. p. 134. 1902.

(5) Kutscher and Seemann. *Ibid*, xxxiv. p. 528. 1902.

(6) von Bergmann and Langstein. Hofmeister's Beiträge vi. p. 27. 1904.

(7) Salvioli. Du Bois Reymond's Archiv. 1880. Suppl. p. 95.

(8) Folin. Amer. Journ. of Physiol. xiii. p. 47. 1905.

(9) Schöndorff. Pflüger's Archiv, lxii. p. 1. 1895.

(10) Jacoby. Hoppe Seyler's Zeitsch. xxx. p. 149. 1900. Lang. Hofmeister's Beiträge v. p. 321. 1904.

(11) Folin. Amer. Journ. of Physiol. xiii. p. 66. 1905.

these for the estimation of the total nitrogen, and two samples of 20 c.c. each were each mixed with 25 c.c. of 20 % tannic acid, containing 5 % of acetic acid, in stoppered bottles: on the next day these were filtered, tested to see that precipitation was complete, and 15 c.c. of the filtrate analysed. The fluids introduced and recovered from the intestine were similarly analysed: the latter was measured, mixed with the washings of the bowel, coagulated by boiling, filtered, evaporated, and made up to a definite volume. The solutions used for absorption consisted of Witte's peptone (5 %) in two experiments: a solution of albumoses prepared by treating Witte's peptone with ammonium sulphate, dissolving the precipitate and dialysing off the ammonium sulphate; such a solution we used in five experiments: and a solution of pancreatic digestion products that gave no reaction with the biuret test; this also was used in five experiments.

The results of the experiments are given in the following Table (p. 468).

In all of these experiments absorption took place, the amount of nitrogen absorbed in the course of from two to three hours corresponding to an amount of proteid that varied from about 2 to 6·5 grm. In every case the total nitrogen in a given volume of blood was diminished, in part at any rate, on account of the water absorbed from the intestine, but in every case the amount of nitrogen present in the form of compounds that are not precipitated by tannic acid was increased, and not diminished. The increase, it is true, is small, but it is constant, and taking into account the fact that the blood was more dilute at the end of the experiment than at the beginning, the increase is somewhat larger than it appears to be. Except in Exp. 7, where the dog was too small to allow the removal of sufficient blood at the beginning of the experiment, double analyses were done in each case, and in four cases only is the second analysis not available through some mishap. The mean between the two results is given in the table, but the divergence of the two results from the mean is at the same time given with the sign ± prefixed. It will be seen that the increase in the figures of the fourth column of blood analyses as compared with the figures in the second column lie in every case outside the limits of analytical error. The difference is therefore a real one, though a small one. In Exp. 12 the difference is seen to be progressive: the dog in this experiment was large enough to give three samples of blood before the final bleeding, and accordingly samples were taken half-an-hour and an hour after the first portion of the peptone solution was run into

TABLE I.

Exp.	Wt. of dog in kg.	Solution introduced: Nature of solution	Solution introduced: Volume in c.c.	Solution introduced: Total nitrogen as c.c. $\frac{N}{10}$ NH_3	Fluid recovered from gut: Time, after the first portion was introduced	Fluid recovered from gut: Amount in c.c.	Fluid recovered from gut: Total nitrogen as c.c. $\frac{N}{10}$ NH_3	Nitrogen absorbed: As c.c. $\frac{N}{10}$ NH_3	Analyses of blood, Before absorption: Total N. in 5 c.c.	Analyses of blood, Before absorption: N. not precipitated by tannic acid in 5 c.c.	Analyses of blood, After absorption: Total N. in 5 c.c.	Analyses of blood, After absorption: N. not precipitated by tannic acid in 5 c.c.	Urine, Excreted during absorption: Vol. in c.c.	Urine, Excreted during absorption: Total nitrogen
1	5·0	cā. 5 % Peptone	185	940	2° 0′	52	585	365	104·5 ±0·1	1·09 ±0·05	101·1 ±0·15	1·35 ±0·025	—	—
2	5·8	Albumose	130	1796	2° 10′	25	1245	551	130·1 ±0·2	1·09 ±0·05	126·9	1·37 ±0·025	17·5	236
3	6·0	Pancreatic digest	164	1760	2° 0′	203	1520	240	125·7 ±0·125	1·2 ±0·05	119·65 ±0·45	1·8	0	—
4	6·5	,,	220	1086	3° 10′	178	594	492	94·75 ±0·15	1·37 ±0·025	83·75 ±0·25	1·91 ±0·05	23·75	125
5	4·8	,,	325	1260	2° 30′	245	965	295	100·05 ±0·07	1·17 ±0·05	97·32 ±0·125	1·78 ±0·05	0	—
6	5·4	,,	220	1148	2° 30′	126	434	714	127·5	1·4 ±0·025	112·4 ±0·2	2·7 ±0·125	1	—
7	3·8	,,	120	528	2° 40′	20	64	464	108·6	1·25	101·85 ±0·15	1·75 ±0	6·5	148·6
8	6·8	Albumose	125	812	1° 50′	<10	390	432	126·95 ±0·35	1·01 ±0·1	115·65 ±0·35	1·43 ±0·05	5·6	196·4
9	7·0	,,	225	1487	2° 45′	40	795	692	141·2	2·58 ±0·05	126 ±0·05	3·04 ±0·05	24	288
10	6·6	,,	300	1170	2° 30′	70	710	460	121·65 ±0·25	1·17 ±0·05	105·07 ±0·4	1·71 ±0·025	1·2	—
11	9·0	,,	175	763	2° 45′	26	540	223	99·9 ±0·1	0·92 ±0·1	87·45 ±0·15	1·26 ±0·075	23·7	124·5
12	12·0	Peptone cā. 5 %	175	885	2° 10′	5	160	725	139·9 ±0·3	1·26 ±0·025	(1) 129·7 ±0·3 (2) 125·8 ±0·6 (3) 118·9 ±0·3	(1) 1·49 ±0·025 (2) 1·64 ±0·025 (3) 1·78 ±0·025	14·7	134·7
13	5·8	Normal saline	200	0	1° 55′	20	12	0	116·82 ±0·1	1·16 ±0·05	90·35 ±0·65	0·6 ±0·1	15·6	142·6

(1) Sample taken half-an-hour, (2) one hour, (3) two hours ten minutes after the introduction of the peptone solution began.

saline solution. The contents were usually deeply stained with blood pigment, and very thick and pasty. On opening the bowel the mucous membrane presented an appearance very far from normal: it was swollen, intensely discoloured in parts, and desquamating: the thick slimy paste removed with the fluid from the bowel was in part composed of desquamated swollen epithelium. This fluid together with the salt solution used for washing the bowel was acidified with acetic acid and boiled. It was then filtered and evaporated down with the addition of a little chalk, again filtered and made up to a definite volume, and two fractions removed for determination of the total nitrogen, two other fractions were treated with tannic acid and, after standing till the next day in stoppered vessels, were filtered, and a part of the filtrate similarly analysed. Samples of the blood taken at the beginning and end of the experiment were examined in the same way.

Exp. A. 160 c.c. of a 10% solution of Witte's peptone introduced into the excised intestine of a dog, in six portions at intervals of a quarter of an hour. The total nitrogen in the peptone solution was equal to 1616 c.c. of decinormal NH_3, and of this 397 c.c. was in the form of compounds that were not precipitated by tannic acid. After two hours perfusion was stopped and 285 c.c. of thick dark fluid were recovered from the bowel. After coagulation by heat the filtrate contained nitrogen equal on analysis to 1810 c.c. of decinormal NH_3, and of this 1400 c.c. were in compounds not precipitated by tannic acid.

Exp. B. This experiment was exactly similar to the last quoted except that the fluid introduced consisted of 150 c.c. of a filtered pancreatic digest containing altogether nitrogen equal to 360 c.c. $\frac{N}{10}$ NH_3, all but 12 c.c. of which was not precipitated by tannic acid. The fluid recovered contained nitrogen in uncoagulable combinations equal to 590 c.c. of $\frac{N}{10}$ NH_3 of which 537 c.c. were in the form of compounds that escape precipitation by tannic acid.

In a series of half-a-dozen experiments, otherwise successful, results similar to these were all that we could obtain by this method: the other experiments therefore need not be described in detail. So we concluded that:

(1) Perfusion of the isolated bowel of dogs even with undiluted dog's blood does not keep the mucous membrane in a normal condition, although to judge by the movements executed it keeps the muscular coats alive.

(2) No demonstrable absorption of nitrogenous substances takes place under these conditions.

(3) Peptone is hydrolysed in the perfused bowel, and apparently

this is the fate too of some of the proteids of the effused blood, since the nitrogen in uncoagulable combinations in the bowel increased in amount during the course of the experiments.

And especially with reference to the interpretation of Salvioli's experiments, we are inclined to think that the conclusions drawn from them are probably wrong. Peptones disappeared from the loops of gut, but to judge from our results it is improbable that it or any derivatives from it were absorbed: probably it was hydrolysed, and was regarded as having been absorbed simply because after hydrolysis the products no longer gave the peptone reactions. Cohnheim has already suggested that the reason why in these experiments the peptone that was missing from the intestine was not found in the blood was that it was acted on by the erepsin after absorption into the epithelium. Our evidence points rather to hydrolytic changes brought about in the lumen of the intestine by enzymes liberated from the cells. In reading the rather short account given of the appearances of the gut at the end of Salvioli's experiments we get the impression that the mucous membrane may have been in no more normal condition than it was in ours. Indeed we cannot understand how it can have been, for diluted blood we found produced a condition worse than undiluted blood, and the blood which he employed was also that of a different species.

We accordingly abandoned the attempt to trace absorption in the isolated intestine and proceeded as follows. The dogs were prepared by being given about a drachm of liquid extract of male fern in the morning and a dose of castor-oil about six hours later. On the next day, no food having been given for 36 hours, morphia and A.C.E. were given and the abdomen opened. The small intestine was divided at the beginning of the jejunum, and the end of the ileum, and vertical tubes were connected with the two ends of the intervening part of the bowel. Through the upper tube the solutions for absorption were admitted in small quantities at a time: through the lower one gas could escape, and into it the peristaltic movements could expel fluid which subsequently would flow back again as the pressure was relaxed. In most cases cannulæ were introduced into the ureters, and tubes leading from these allowed the urine to be collected and measured. The abdominal incision was then stitched together, allowing only the tubes leading from the intestine or the ureters to project between the sutures. A sample of the animal's blood was drawn off from the carotid artery before any solution was put into the bowel and another at the end of the experiment. Two samples of 5 c.c. each were taken from each of

tated by tannic acid to the total amount of nitrogen absorbed from the intestine. For this purpose we have reckoned that the volume of blood in the animals' bodies was 75 c.c. for each kilogram of body weight, and as far as it is possible to do so we have made allowance for the blood drawn at the beginning of the experiment and for the dilution of the blood, of which we have evidence in the diminution in the total nitr of the blood, and also in hæmoglobinometric determinations which taken colorimetrically in the different samples.

We find in this way that on an average about one-seventh of nitrogen absorbed is accounted for by the increase of nitrogen in the filtrate from the tannic acid precipitations. In one experiment it amounted to one quarter, but in two to less than a tenth.

As to the rest of the nitrogen absorbed it is impossible from the data in these experiments to say how much of it corresponds to the nitrogen excreted in the urine. In most of the experiments, it is true, the nitrogen excreted in this way during absorption was considerable in amount. A dog under anæsthesia very frequently secretes no urine, especially if it has been purged and has not had food for 24—36 hours previously. In four of our experiments that was practically the case. In others in which we waited after introducing the cannulæ into the ureters in order to be able to compare the rate of nitrogen excretion before and during absorption we found that this rate was considerably increased during absorption. Thus in Exp. 4 no urine was excreted in the hour preceding the introduction of fluid into the intestine, and then the flow began and amounted to nearly 24 c.c. in 3 hours 10 mins., which contained nitrogen equal in amount to about one-quarter of that absorbed in this time. In Exp. 2 the rate of nitrogen excretion for 1 hour 40 mins. before the absorption began was 10·6 c.c. $\frac{N}{10}$ NH_3 in 10 mins. and afterwards during absorption averaged 19·6 c.c. in 10 mins., the increase in the urinary volume in the same time being much less than this. In Exp. 9 there was no excretion till 50 mins. after the first fluid was run into the bowel, and then in the next two hours the urine amounted to 24 c.c. containing nitrogen equal to more than 40 % of that absorbed. In Exp. 11 the urine excreted in the time preceding absorption was very poor in nitrogen compared with the later samples: in 5 c.c. the nitrogen was equal to 11 c.c. $\frac{N}{10}$ NH_3 as compared with 26·3 c.c.: and the total amount of nitrogen excreted during absorption amounted to 55 % of

the absorbed nitrogen. But although in the majority of cases the absorption of nitrogenous substances was accompanied by active nitrogen excretion there were, as is usual, irregularities in the excretory functions, and in any case it is impossible to say to what extent the nitrogen excreted during absorption was the same as that which was absorbed.

We are left therefore unable to trace the greater part of the absorbed nitrogen. We endeavoured to determine whether it was lodged in the liver; but we found considerable difficulty in the attempt, and we are not very satisfied with the results. For this purpose the left lobe of the liver was ligatured and removed at the time of the operation, and compared with the remainder of the liver at the end of the experiment. Two small pieces were cut off the left lobe, weighed, and the total nitrogen determined in these: two larger portions were cut up fine and dried at 105° C.: and finally two portions of about 10 grm. each after being weighed were treated with anhydrous sodium sulphate, and after dehydration by this salt powdered and boiled in alcohol[1], the dry residue after evaporation of the alcohol was extracted with boiling water repeatedly, the extract filtered and made up to the same volume in each case, of which a fraction was taken for nitrogen determination. The results obtained are given in the following table.

TABLE II. *Analyses of the Livers.*

		Total nitrogen in 1 gr. fresh tissue in c.c. $\frac{N}{10}$ NH_3	Water in 100 gr. of fresh tissue	Uncoagulable nitrogen in 1 gr. fresh tissue	Gain in total nitrogen per 1 gr. solids	Gain in uncoagulable nitrogen per 1 gr. solids
Exp. 6	Left lobe	25·3 ± 0·1	70·4 ± 0·1	1·695 ± 0·025		
	Right lobe	26·35 ± 0·05	71·03 ± 0·13	2·10 ± 0·1	1·6	0·45
Exp. 7	Left lobe	23·35 ± 0·05	73·75 ± 0·25	1·30 ± 0·01		
	Right lobe	23·65 ± 0·05	73·6 ± 0·1	1·60 ± 0·08	0·28	0·28
Exp. 8	Left lobe	19·55 ± 0·05	62·15 ± 0·25	1·12 ± 0·02		
	Right lobe	19·5 ± 0·4	62·20 ± 0·3	1·39 ± 0·05	0·05	0·29

In each case we found an increase in the extracted nitrogen, though it was very small: in Exp. 7 reckoned for the whole liver it would account for 65 c.c., which would be about 15 % of the absorbed nitrogen. But it is noticeable that the figures show that this increase in uncoagulable nitrogen was in one case the same as the increase in

[1] This method, based on the suggestions of Pinkus (This *Journal*, XXVII. p. 57. 1901), has been extensively used by Dr Schryver, who kindly communicated to us the results of his experience with it.

the bowel, in addition to the one at the end of the experiment. Each specimen of blood removed during absorption is more dilute than the last, but contains more nitrogen after precipitation with tannic acid. In Exp. 13 the conditions were the same in every respect except that no nitrogenous substances were introduced, but a normal solution of sodium chloride used instead. In this case the blood becomes more dilute as in all the other experiments, but it also contains less of the nitrogen of the kind that we are concerned with, the diminution of this nitrogen is in fact more pronounced than that of the total nitrogen. This no doubt is due to the diuretic action of the absorbed salt solution causing the kidneys to clear the blood of excretory products. This experiment therefore serves as a control of the others, and justifies us in associating the absorption of nitrogenous substances with the increase of nitrogenous compounds in the blood, which are not precipitated by tannic acid. But this is not the only control that we have. In the perfusion experiments referred to earlier in which we failed to get the intestine to absorb nitrogenous substances, in all five experiments in which the blood was analysed, the ratio of this nitrogen to the total nitrogen was either unchanged or diminished.

Our results therefore are quite clear on this point, that during absorption of nitrogenous substances, whether pure albumoses, Witte's peptone, or digestion products that do not give the biuret reaction, the nitrogenous substances in the blood that are not precipitated by tannic acid are definitely increased in amount, though only to a small extent.

We then tried to determine something as to the nature of the nitrogenous combinations in the blood that were increased during absorption. In the first place in view of the well-known results obtained by Nencki and his collaborators, which showed that the amount of ammonia in the portal blood during digestion of proteids was greater than that in the systemic blood, as well as the experiments of Lang and others on the denitrification of amido-acids in the intestine and liver and other organs, we were interested in the variations of ammonia in the blood in our experiments.

We made use of the method of Folin[8] which consists in blowing air through the blood at a temperature of about 25° C. and leading the air through titrated sulphuric acid. We tested the method as we practised it and found that 25 c.c. of blood, which by itself gave 0·2 c.c. of decinormal ammonia, gave after adding 12·5 c.c. of decinormal ammonia solution under the conditions in which our estimations were made 12·2 c.c. In Exp. 9 25 c.c. of the blood at the beginning con-

tained ammonia equal to 0·55 c.c. $\frac{N}{10}$ sulphuric acid, and at the end the same volume of blood contained ammonia equal to 0·68 c.c. of the same acid. The ammonia in 5 c.c. of blood therefore increased by an amount represented by less than 0·03 c.c. In 5 c.c. of blood the nitrogen which was not precipitated by tannic acid increased, if allowance be made for the dilution of the blood, by an amount equal to 0·74 c.c., so that we were able to account for only about 4 % of the increase in nitrogen soluble in tannic acid by means of the amount of ammonia that we found. In Exp. 11 there was less ammonia detected in the blood after the absorption than before. We conclude therefore that the nitrogenous compounds increased in the blood during absorption do not include ammonia in sufficient quantity to account for the phenomenon.

Urea was the next nitrogenous compound to think of, and in two experiments we compared the amount of urea in the blood before and after absorption, using the method of Pflüger and Schöndorff [9].

We tested the range of accuracy of the method in our hands in the following experiments, 50 c.c. of blood to which 4 c.c. of a solution of urea of known nitrogen content were added gave us on distillation 21·55 c.c. $\frac{N}{10}$ NH_3 more than 50 c.c. of the same blood to which 4 c.c. of water had been added. The amount of urea added should have theoretically yielded 21·72 c.c. In a second test carried out with about a tenth of the amount of urea our result was 2·05 c.c. instead of the theoretical 2·23 c.c.

Proceeding in this way with 50 c.c. of the blood obtained in Exp. 10 we found there was an increase in the urea in the blood during absorption which reckoned on 5 c.c. of blood accounted for 0·315 c.c. $\frac{N}{10}$ NH_3. The whole amount of nitrogen increase in this amount of blood after allowing for the change in concentration was only 0·7 c.c.: so that in this experiment the increase detected in the urea accounts for nearly half the whole increase in nitrogen soluble in tannic acid. In the Exp. 11 the increase in urea accounted for nitrogen in 5 c.c. of blood equal to 0·21 c.c. $\frac{N}{10}$ NH_3, which is again nearly half the whole increase of nitrogen soluble in tannic acid that we detected.

To take another point, we have calculated the ratio borne by the increase in the amount of nitrogen contained in the blood not precipi-

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On the Filtration of Crystalloids and Colloids through Gelatine: with special reference to the behaviour of Hæmolysins.

By J. A. CRAW, British Medical Association Research Scholar.

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The current controversy between Ehrlich (1898, 1903) and Arrhenius and Madsen (1902, 1904) on the physical chemistry of the neutralisation of toxins by their specific antitoxins led the author (IV, 1905) to an investigation of the relations existing between the toxin for red blood corpuscles secreted by *B. megatherium* and its specific antiserum. One of the methods adopted consisted in the filtration of mixtures of megatherium hæmolysin and antilysin through gelatine. The lysin was found to pass into and even through the gelatine, whereas the antilysin was retained, and by means of the delicate blood test for free lysin it was possible to demonstrate that the two substances, on mixing in any proportions, do not completely neutralise each other. These observations indicated that Ehrlich's views on the toxin-antitoxin reaction required considerable modification, but a closer investigation showed that the hypothesis advanced by Arrhenius and Madsen agreed even less with the experimental facts. On the other hand, the results were in entire harmony with the views advanced by Landsteiner (1903) and Bordet (1903), which have been supported by Nernst (1904) and Craw (1905) that the toxin is adsorbed by the antitoxin much as a dye is by a tissue. As this conclusion may considerably modify current ideas on the nature of the reaction and the constitution of toxins in general, it seemed advisable to inquire further into the physical chemistry of filtration through gelatine.

The present communication contains data of the filtration of various crystalloidal and colloidal solutions, including megatherium lysin, through various percentages of gelatine, under constant and variable pressures.

The work was partly carried out as Research Student at the Lister Institute of Preventive Medicine and was completed at the London Hospital Medical College by the aid of a scholarship from the British Medical Association.

Previous Work on the Gelatine Filter.—The gelatine filter was introduced by C. J. Martin (1896), and consists of a Pasteur-Chamberland candle, the pores of which are filled with solid gelatine. The filter is fitted into a gunmetal jacket or filter case, which serves to hold the liquid to be filtered, and the upper end of the closed filter case is connected with a supply of air at a pressure of 30 to 100 atmospheres, which is used to force the liquid

through the gelatine. From his observations with "wet" gelatine filters, *i.e.*, filters containing gelatine from which the normal content of water had not been removed by drying in air or otherwise, Martin* concluded that gelatine was impermeable to colloidal substances such as globulin, albumin, glycogen, and soluble starch, but partially permeable to albumoses and dextrin, and completely permeable to solutions of crystalloids, *e.g.*, urea and dextrose. It seemed, then, as if the gelatine filter was an instrument destined to play an important part in the investigation of physiological fluids.

Martin and Cherry (1898) applied the filter to the investigation of the course of the reaction occurring between diphtheria toxin and antitoxin, and likewise to the reaction of snake venom with antivenene, the toxin and venom being filter-passers, whereas the anti-bodies† were retained.

From these experiments it seemed as if the toxin was completely neutralised by the antitoxin, but further investigation of the mechanism of gelatine filtration shows that no such absolute conclusion can be drawn. E. Waymouth Reid (1901) showed conclusively that crystalloids do not pass "wet" gelatine filters in unaltered concentration, and that although filters which had been dried to constant weight in dry air allowed certain crystalloidal solutions to pass unchanged, yet dextrose and sodium oleate‡ were partially retained. Further, he found that the filtrate from serum§ had not the same composition as the proteid-free serum, and that the residual fluid left in the filter case had a much higher concentration of organic substances of non-proteid character than either the original serum or the filtrate. The Martin filter is not, therefore, a simple means of separating crystalloids from colloids. One must not, however, under-estimate its value as an instrument for the analysis of physiological fluids, for although the filter shows considerable differences in permeability to various crystalloidal substances, I find (IV, 1905) that these and inferior colloids are, on the whole, retained to a small extent compared with typical colloids. The partial retention of filter-passers has an important bearing on the conclusions to be drawn from gelatine filtrations, for, if the concentration of the filter-passer to be tested for be small in the original fluid introduced into the filter, the gelatine may retain practically the whole amount and the filtrate contain only a quantity below the experimental error of observation. This was found to be the case for neutral mixtures of megatherium lysin and antilysin, and mixtures

* *Loc. cit.*

† *Cf.* also Brodie (1897) (1900).

‡ Krafft (1902) considers soap solutions, such as sodium oleate, to be colloidal.

§ *Cf.* also Starling (1899).

containing excess of antilysin, by the author;* the filtrates showed no hæmolytic power, whereas the gelatine had stored up a considerable amount of free megatherium lysin. In this light Martin and Cherry's observations on diphtheria toxin and snake venom, mentioned above, are in entire agreement with my results for megatherium lysin, the free toxin and venom of the neutral mixtures being probably stored up in the gelatine of their filters.

On "Wet" and "Dry" Gelatine Filters.—Martin and others used the filters in the "wet" state, *i.e.*, shortly after solidification of the gelatine in the pores of the candle, and, therefore, containing a considerable quantity of water, part of which on filtration will pass into the filtrate and so dilute the substance filtered. To get rid of this difficulty, E. W. Reid removed part of the water by drying Martin candles to constant weight in a current of dry air. It seemed to me, however, that by drying the gelatine another difficulty might be introduced, viz., a change in the size of the pores, which would render observations with "dry" filters of doubtful value.

Method.—The rate of filtration of water through freshly prepared wet filters was observed during one hour and compared with the rates during a similar period, of filters which had been partially dried by standing in dry air for 10 hours, 24 hours, and 3 days.

Rates of Filtration.—The freshly prepared wet filters gave fairly uniform rates, and allowed from 0·5 to 2 c.c. of water to pass per minute at 100 atmospheres pressure, the concentration of the gelatine in the pores being 9 per cent. and the temperature 10° to 15° C. Under the same conditions the partially dried filters allowed from 5 to 20 c.c. to pass per minute at the beginning of the filtration, but the rate rapidly decreased. On reducing the applied pressure to about one atmosphere, and allowing the water to flow through a partially dried filter at the rate of about 1 to 2 c.c. per minute, the filter gradually tightened, so much that after the passage of 30 to 50 c.c. a pressure of 100 atmospheres was necessary to maintain the same rate. The longer the filters were dried the more marked was the porosity and the greater the amount of fluid which had to be pressed through before the filter tightened.

Conclusions.—On partially drying gelatine filters the gelatine shrinks, and air passages are produced of greater diameter than the water passages of a wet filter. The wide dry filter passages at first offer a free passage to the fluid filtered, and no material change is to be expected in the percentage composition of the latter. The filter gradually changes in character until ultimately, a wet filter is obtained with pores of similar dimensions to those of a freshly prepared wet filter. The various fractions of filtrate from a

* *Loc. cit.*

"dried" filter are not subject to the same conditions of filtration and are not, therefore, comparable. Wet filters, on the other hand, show a much greater constancy in rate of filtration, and are, therefore, to be preferred.

On the Preparation of the Filters.—To overcome the difficulty of dilution of the filtrates by the water of wet filters, the gelatine to be used in filling the candles was in several cases dissolved in the fluid to be filtered. Thus the filtration of 0·8 per cent. sodium chloride and of megatherium lysin took place through 9 per cent. gelatine which had been dissolved in 0·8 per cent. sodium chloride, and 1·54 per cent. potassium iodide was filtered through 11 per cent. gelatine in 1·54 per cent. iodide. A Pasteur-Chamberland candle of size B, which had in the majority of cases been heated for 10 hours at least in a muffle furnace to remove organic matter, was fixed into a brass socket with "Faraday" cement and fitted concentrically into the internally tinned gunmetal jacket or "filter case." After thoroughly washing through with about 400 c.c. of hot water and 250 c.c. of the solution of French gelatine to be used in forming the filter, at a temperature of 37° C., the gelatine at 30° C. was slowly passed through the cooling filter at an air pressure just sufficient to cause about 1·5 c.c. or 15 drops to filter per minute. The filtration was continued until no trace of air bubbles was visible in the drops, after which a further 50 c.c. were allowed to pass. During the final filtration the upper surface of the gelatine solution in the filter case was not allowed to descend below a level 3 cm. above the crown of the candle. The filter was then removed, drained, placed in the neck of a flask containing a little water, and kept in the ice chest at 6° to 8° C. until required. If the filter so prepared had an obviously thick skin of gelatine upon its surface, this was removed by immersing in the gelatine solution at 30° C. and draining as before. In the comparative experiments with different concentrations of gelatine, the filters were treated in the manner last described to get membranes of approximately equal thickness. Only those filters were used which allowed less than 2 c.c. or about 20 drops per minute to pass at 100 atmospheres pressure and 10° to 15° C., and the majority of filters allowed only about 0·5 c.c. per minute to pass under these conditions. After use the candles could be washed out with water at 50° C., dried, heated in a muffle furnace, and on refilling with gelatine again gave reliable filters.

Filtration of a Typical Crystalloid, Sodium Chloride, through 9 *per cent. Gelatine.*

Sodium chloride was chosen on account of its characteristic crystalloidal properties and the important *rôle* it plays in physiological fluids. Further,

in a previous communication,* it was found that the filtrates from megatherium lysin were strongly hæmolytic, and it was necessary to determine if this hæmolysis was due in any degree to change in the concentration of sodium chloride.

Method.—The filters used contained 9 per cent. gelatine in 0·8 per cent. NaCl, and about 50 c.c. of 0·8 per cent. NaCl were pressed through at 100 atmospheres and 10° to 12° C., the filtrates being collected in fractions of about 4 c.c. The NaCl content of 1 c.c. of the fractions of filtrate, the residual liquid left in the filter case and the original fluid, was determined by titration with 1/100 normal silver nitrate, using potassium chromate as an indicator. The hæmolytic powers of the same fluids were determined in these and all other experiments in this paper, unless otherwise stated, by mixing 1 c.c. of the respective fractions with 2 c.c. of 2·5 per cent. guinea-pig's red corpuscles which had been washed and suspended in 0·8 per cent. NaCl. The mixtures were heated to 37° C. for three hours, well shaken every 30 minutes, and allowed to sediment generally about 12 hours in the ice chest at 6° to 8° C.

The intensity of tint of the supernatant fluid was then determined by comparison with the scale of a von Fleischl hæmoglobinometer which had been standardised by a blood solution of known content. Complete hæmolysis is indicated by the index (100).

Examination for Sodium Chloride and Hæmolytic Power.—Experiment No. 2, Table I, represents one out of four similar experiments.

Original Fluid: 0·81 per cent. NaCl given in the table as Orig. (100).

Filtrates: The relative concentrations of the 1st, 4th, and 14th fractions were (47·1), (89·5), and (99·2) respectively.

Residual Fluid: When tested immediately after decompression had the value (100) exactly, but, on allowing to stand 12 hours in the filter case, showed the relative concentration (105).

Hæmolytic Powers: No hæmolysis was obtained under the standard conditions with either filtrates, residual fluid, or the original saline.

Conclusions.—The typical crystalloid, sodium chloride, is markedly retained on filtration through gelatine which originally contained the same concentration of saline as the fluid filtered. The salt taken up by the gelatine is expressed or diffuses into the residual fluid after decompression. The diminution in salt concentration in the filtrate is insufficient to cause hæmolysis under the standard conditions.

* *Loc. cit.*

Continued Filtration of Sodium Chloride through 9 per cent. Gelatine.

As the last fraction of filtrate in No. 2 did not quite reach (100), it seemed desirable to ascertain whether on continued filtration this value could be obtained.

Method.—The residual fluid of No. 2, which had stood in the filter case

Table I.

	Sodium chloride, per cent.						Megatherium lysin, H.Is.				
No. ...	1.	2.	2A.	2B.	3.		4.	5.	6.	7.	8.
P. c. ...	0·88.	0·81.				HBū.	15.	11.	9.	7·5.	S.
Successive fractions of filtrate.	23·9	47·1	101·5	97·4	16·7	0·2	0·7	0·5	0·0	0·5	0·0
	31·4	55·1	103·3	97·8	41·7	5·8	0·5	0·0	0·0	5·0	3·5
	39·0	76·1	99·6	96·0	47·8	60·4	0·0	2·3	0·0	17·7	10·7
	49·0	89·5	101·1	93·1	56·9	74·0	0·0	5·1	3·7	25·0	17·8
	56·0	95·3	99·6	94·2	65·6	85·4	0·0	5·1	27·8	34·6	21·4
	64·1	96·7	97·6	93·5	70·6	88·5	0·0	7·1	46·3	45·1	25·0
	71·1	97·6	100·7	95·7	76·8	88·5	0·0	8·6	33·9	48·8	28·5
	76·1	97·6	97·6	94·2	80·4	90·6	0·0	8·6	47·8	48·8	38·5
	79·2	97·6	—	94·2	83·1	90·6	0·0	8·6	47·0	48·8	32·1
	81·7	99·2	—	96·0	85·0	90·6	0·0	12·3	47·0	48·4	32·1
	83·3	99·6	—	94·9	86·3	90·6	0·0	12·8	45·6	48·4	35·7
	112·6	100·0	—	96·8	—	—	2·3	8·0	47·0	49·3	35·7
	107·8	99·6	—	96·4	—	—	3·7	10·0	46·3	48·8	50·0
	107·8	99·2	—	96·4	—	—	10·0	—	—	48·4	50·0
	98·4	—	—	96·4	—	—	—	—	—	—	50·0
	96·2	—	—	—	—	—	—	—	—	—	50·0
	93·7										
Res.	134·9	100·0	100·0	100·0	110·0	125·0	85·0	62·9	55·6	52·7	67·8
	c.c. 20	c.c. 44	c.c. 65	c.c. 40	c.c. 45	—	c.c. 47	c.c. 45	c.c. 34	c.c. 46	c.c. 31
Orig.	100·0	100·0	100·0	100·0	100·0	100·0	50·0	50·0	50·0	50·0	50·0
	c.c. 100	c.c. 112	c.c. 112	c.c. 120	c.c. 105	—	c.c. 110	c.c. 105	c.c. 100	c.c. 110	c.c. 105

overnight, was poured out, and the filter case refilled with 112 c.c. of saline, of which 40 c.c. in 5 c.c. fractions were pressed through.

Examination for Sodium Chloride.—Experiment No. 2A, Table I.

Original Fluid: 0·81 per cent. NaCl = Orig. (100).

Filtrates: The 1st and 2nd fractions contained (101·5) and (103·3) respectively, and those succeeding showed values varying about (100).

Residual Fluid: Titrated immediately after decompression (100), after 12 hours in the filter case (110).

Conclusion.—On renewing filtration the filtrate may contain a higher percentage of salt than the original fluid, due probably to the temporary decompression, for on continued recompression the concentration tends to become less than (100).

Continued Filtration of Sodium Chloride with Megatherium Lysin and Antilysin through 9 per cent. Gelatine.

In a former paper,* mixtures of lysin and antilysin were pressed through gelatine which had been tested for tightness by the filtration of saline. The redistribution of salt under these circumstances was now determined.

Method.—A filter through which 10 c.c. of 0·81 per cent. NaCl had been pressed, the original gelatine being 9 per cent. in 0·81 per cent. NaCl, was decompressed, the residual salt solution removed, and the filter case refilled with 120 c.c. of a nearly neutral mixture of equal volumes of a fluid megatherium lysin and 5 per cent. antilysin in saline, of which 75 cc. in 5 c.c. fractions were pressed through.

Examination for Sodium Chloride.—Experiment No. 2B, Table I, is one of two practically identical filtrations.

Original Fluid: Contained 0·81 per cent. NaCl = (100). Hæmolytic Index (16·8).

Filtrates: As in No. 2A the 1st and 2nd fractions were the most concentrated in NaCl, but they did not reach (100), and the final fraction was (96·4). The average hæmolytic index of the whole filtrate was (0·5).

Residual Fluid: The results were entirely similar to those of No. 2A as regards NaCl. Hæmolytic Index (38·6).

Conclusion.—The redistribution of salt is insufficient to give a trace of hæmolysis under standard conditions.

Temporary decompression even for a few minutes allows a higher percentage of salt to pass into the filtrate than would be obtained by constant pressure.

Filtration of Sodium Chloride with Butyric Acid through 9 per cent. Gelatine.

Butyric acid in saline acts as a very strong hæmolysin, and, as it possesses crystalloidal properties, it appeared to be of interest to compare its filtration phenomena with those of megatherium lysin in saline. Further, butyric

* *Loc. cit.*

acid greatly diminishes the surface tension of saline against air, and the possibility of a change in the surface forces between saline and gelatine formed a second point of interest, which is discussed towards the end of this paper.

Method.—A solution of 0·81 per cent. NaCl containing 4·8 per cent. butyric acid was pressed through 9 per cent. gelatine in 0·81 per cent. NaCl at 100 atmospheres. The content of the fractions of filtrate, etc., in NaCl was determined as before, and the butyric acid was estimated by titration with standard sodium hydrate, using phenol-phthalein as indicator. The hæmolytic powers of the various fluids were also determined by the time taken for 1 c.c. of the fractions to hæmolyse completely 2 c.c. of a 2·5 per cent. suspension of guinea-pig's corpuscles in 0·8 per cent. NaCl at 16° C.

Examination for Sodium Chloride.—Experiment No. 3, Table I. The redistribution of salt was qualitatively similar to that which had been obtained in the absence of butyric acid. The amount passing into the filtrate was, however, smaller.

Examination for Butyric Acid and Hæmolytic Power.—Original: 4·8 per cent. butyric acid = (100) HBū. Hæmolytic time = 8 minutes.

Filtrates: The 1st and 2nd fractions contained (0·2) and (5·8) HBū respectively. The 1st fraction agglutinated the test blood strongly, and the 2nd hæmolysed completely in 50 minutes. The 8th to the 11th fractions contained (90·6) HBū, and the hæmolytic time was 12 minutes.

Residual Fluid: Concentration (125) HBū. Hæmolytic time, 5 minutes.

Conclusions.—Butyric acid is retained by gelatine to a considerable extent. The gelatine appears to retain more sodium chloride in the presence of butyric acid.

Filtration of Sodium Chloride through 9 per cent. Formalised Gelatine.

It seemed probable, from the marked change which takes place in gelatine on being exposed to formic aldehyde, that such gelatine would show a different permeability to that already found for ordinary gelatine. On the other hand, the possibility of investigating physiological fluids in an apparatus which could be thoroughly and easily sterilised by means of "formalin" might recommend the use of formalised gelatine filters.

Method.—A filter containing 9 per cent. gelatine in 0·8 per cent. NaCl was fitted into the filter case, and the latter filled with a solution of 0·8 per cent NaCl containing 10 per cent. of commercial "Formalin." A few cubic centimetres of fluid were pressed through, and the remainder allowed to stand in the filter case overnight. The solution was then removed, and, after about 12 hours, 100 c.c. of 0·88 per cent. NaCl were placed in the filter case, and pressed through in fractions of 4 to 5 c.c.

Examination for Sodium Chloride.—Experiment No. 1, Table I.

Original Fluid: 0·88 per cent. NaCl represented as Orig. (100).

Filtrates: No formaldehyde or formic acid could be detected. From the 1st to the 11th fraction the NaCl gradually increased from (23·9) to (83·3), at 100 atmospheres filtration pressure. At this point the pressure was removed and filtration resumed after an interval of eight hours. Six fractions of 5 c.c. each were then removed, and gave values decreasing from (112·6) to (93·7).

Residual Fluid: Tested immediately after the last decompression contained (134·9).

Conclusions.—Formalised gelatine containing sodium chloride retains sodium chloride from a solution, on filtration, to a greater extent than ordinary gelatine. The decompression in this case also leads to the immediately following filtrates having a higher concentration of salt than the original fluid, but the effect is more marked than with ordinary gelatine. The concentration of the residual fluid is also much greater than with ordinary gelatine.

Filtration of Megatherium Lysin Through Various Percentages of Gelatine.

Megatherium lysin diffuses slowly compared with crystalloids in general,* and is probably of semi-colloidal character. It seemed probable that a substance of this type would be considerably more affected by a change in the concentration of gelatine than a good filter-passer such as sodium chloride. Hardy's (1899) work on gelatine pointed also to the possibility of considerable change in the structure of the jelly at about 7 per cent. gelatine, which might lead to markedly different degrees of permeability between 7 and 15 per cent gelatine filters.

Method.—Filters containing 15, 11, 9 and 7·5 per cent. gelatine in 0·8 per cent. NaCl were tested for tightness with 0·8 per cent. NaCl, of which 10 c.c. were pressed through in each case to ensure a concentration of NaCl in the succeeding filtrates of about 0·8 per cent. The fluid examined was the hæmolytic filtrate from a broth culture of *B. megatherium* diluted with an equal volume of 0·8 per cent. NaCl.

Filtration was carried through at a pressure of 100 atmospheres and a temperature of 10° to 12° C. and the filtrate was collected in fractions of about 4 c.c.

Examination for Lysin.—Table I.

Original Fluid.—The hæmolytic index (see above) was (50).

* *Cf.* Craw (IV, 1905).

Filtrates: Experiments Nos. 4, 5, 6 and 7, Table I, show the hæmolytic values of the succeeding fractions through 15, 11, 9 and 7·5 per cent. gelatine respectively. The average indices for the whole filtrates were in the order given above (1·5), (6·9), (31·7), and (36·9).

In Experiment No. 8, Table I, 50 c.c. of 20 per cent. horse serum in 0·8 per cent. NaCl had previously been passed through a 7·5 per cent. gelatine filter. In this case the permeability to lysin was slightly less than that of a similar filter without serum, the average indices of the filtrates being (30·6) and (36·9) respectively.

Gelatine: On melting out the gelatine, after filtration, at 37° C., and mixing with the test blood, the latter was in all cases rapidly and completely hæmolysed, index (100). Control experiments showed that saline on being pressed through gelatine which had been used to filter lysin became strongly hæmolytic. The original gelatine had no hæmolytic effect in the standard time.

Residual Fluids: The residual fluids in all cases showed average indices which were higher than that of the original fluid. The lowest portions of residual fluid had generally higher indices than the portions towards the upper surface. With rising concentration of gelatine the hæmolytic power increased, thus with 7·5 per cent. the residue had an index (52·7) whereas with 15 per cent. it was (85).

Conclusions.—Megatherium lysin is retained to a greater extent than sodium chloride, and more is retained with higher concentrations of gelatine. The residual fluids have higher concentrations than the original and the concentration is greater with higher percentages of gelatine. Diffusion and expression of lysin from the gelatine into the residual fluid are insufficient to account for the increased concentration immediately after decompression, and it seems as if the water of the original fluid could pass into the gelatine more readily than the lysin.

It seems possible, under the conditions of preparation, that during the draining of the filters from the various percentages of gelatine at 30° C., the filters with the higher concentrations would retain thicker surface layers of gelatine and so exaggerate the differences in permeability.

Filtration of a Typical Colloid, Ferric Hydrate, through 11 *per cent. Gelatine under Varying Pressure.*

As ferric hydrate in colloidal solution shows no appreciable tendency to diffuse, any redistribution of the colloid by the gelatine filter must be explained on some other basis than that of diffusion.

Method.—A 5-per-cent. solution of colloidal ferric hydrate was prepared

by adding ammonium carbonate to ferric chloride and dialysing for three weeks; it gave no trace of red coloration with potassium thiocyanate, but showed the characteristic reaction of colloidal ferric hydrate, viz., a slight yellowish precipitate. The filter used contained 11 per cent. gelatine which had been dialysed for 24 hours. The content of the fractions of filtrate, etc., in iron, was determined by converting the hydrate into chloride and estimating colorimetrically with potassium thiocyanate.

Table II.

Successive fractions of filtrate.	Colloidal ferric hydrate.			Neutral red.			Iodine in potassium iodide.			
	Vol. in c.c.	Pressure in atmos.	Percentage Fe.	Vol. in c.c.	Pressure in atmos.	Percentage N.R.	Vol. in c.c.	Pressure in atmos.	Percentage I_2.	Percentage KI.
	4	100	0·0	4	100	0·0	1	100	5	68·8
	4	100	0·7	4	100	2·3	4	100	8	69·9
	4	100	0·6	4	100	0·04	4	100	17	70·9
	4	100	0·4	3	20	0·03	4	100	19	69·9
	4	100	0·4	10	100	0·30	4	100	21	69·9
	4	100	0·4	10	100	0·78	4	100	23	69·9
	4	100	0·1	10	100	0·90	4	100	25	68·3
	4	100	0·0	10	100	1·17	1	15	20	69·9
	4	100	0·0	10	100	3·00	5	100	33	71·5
	1	100	0·0	10	100	3·20	5	100	33	73·0
	0·5	20	250·0	20	100	4·68	4	100	35	72·0
	1	100	4·75	—	—	—	0·75	0	0	43·0
	2	100	0·9	—	—	—	0·75	100	25	93·5
	3	50	0·7	—	—	—	6	100	37	90·5
	0·5	15	66·6	—	—	—	6	100	50	86·0
	0·75	50	1·0	—	—	—	1	0	1	34·4
	0·25	30	0·6	—	—	—	5	10	25	94·6
	0·5	20	0·3	—	—	—	2	100	30	92·5
	0·75	10	0·1	—	—	—	3	50	30	96·8
	0·25	50	0·2	—	—	—	4	100	30	91·4
	1	100	0·2	—	—	—	5	50	30	85·0
	5	15	0·4	—	—	—	5	25	30	82·8
	4	100	0·4	—	—	—	5	25	25	79·5
	0·5	15	0·4	—	—	—	5	25	25	73·1
	4	100	0·4							
Res.	50	—	180·0	20	—	18·75	35	—	30	109·7
Orig.	120	—	100·0	120	—	100·0	120	—	100	100·0

Examination for Ferric Hydrate.—Table II gives the relations obtained in one out of three similar experiments.

Original Fluid: Five per cent. ferric hydrate = (100) Fe.

Filtrates: The 1st and 2nd fractions contained (0·0) and (0·7) respectively, and in the succeeding fractions the amount diminished until in the 8th no

trace could be found at 100 atmospheres filtration pressure. On suddenly diminishing the pressure to 20 atmospheres the liquid percolating through was very intensely coloured and gave the value (250). Re-establishing the pressure of 100 atmospheres, the filtrates became less and less intensely coloured (4·75) and (0·9). A drop in the pressure to 50 atmospheres did not materially change the concentration, but a further drop to 15 atmospheres gave a filtrate with the value (66·6). Increasing the pressure to 50 atmospheres caused the filter to tighten once more with respect to the hydrate, and on gradually decreasing the pressure to 10 atmospheres the filter remained tight. In this condition sudden variations of pressure from 100 to 15 atmospheres had but a slight effect on the permeability of the filter.

Residual Fluid: The colour was more intense than that of the original fluid and its content was (180) Fe.

Conclusions.—The gelatine fifter is slightly permeable to the typical colloid ferric hydrate, but at constant pressure the permeability decreases as the filtration proceeds. The permeability is increased enormously by suddenly diminishing the pressure, but is not much affected by a gradual diminution. After gradual diminution of pressure a filter is obtained which does not markedly change in permeability on suddenly varying the pressure. The high concentration of the residual fluid is probably due to the water penetrating the gelatine easily, whereas the ferric hydrate is largely left on the surface of the gelatine, where it forms a concentrated solution of higher specific gravity than the rest of the fluid and so gives rise to convection currents which cause it partly to mix with the remainder of the fluid in the filter case. The candle retains a skin of colloidal ferric hydrate, and the filter most probably at the beginning of the filtration acted as a simple gelatine filter, but subsequently as a compound ferric hydrate gelatine filter.

Filtration of a Staining Colloid, Neutral Red, through 11 *per cent. Gelatine under Varying Pressure.*

Preliminary experiments with horse serum and soluble starch showed qualitatively similar effects to those obtained with colloidal ferric hydrate, and it seemed probable that the majority of colloidal solutions would behave in a similar way on filtration through gelatine.

It seemed probable, however, that those colloidal solutions which stain gelatine would show considerable difference in behaviour.

Method.—A 0·5-per-cent. solution of neutral red in distilled water was pressed through 11 per cent. dialysed gelatine and the content of the fractions of filtrate, etc., determined colorimetrically.

Examination for Neutral Red.—Table II.

Original Fluid: 0·5 per cent. neutral red = (100) N.R.

Filtrates: The 1st, 2nd and 3rd fractions showed (0·0), (2·3) and (0·04) respectively. A sudden diminution in pressure from 100 to 20 atmospheres caused a slight decrease in the value of the filtrate (0·03). On raising the pressure once more to 100 atmospheres, the succeeding fractions gradually increased in content up to (4·68) N.R.

Residual Fluid: The colour intensity was much diminished and indicated only (18·75) N.R.

Conclusions.—The gelatine filter is slightly permeable at the beginning of filtration to neutral red, but the permeability decreases, as in the case of colloidal ferric hydrate, on continued filtration at constant pressure. Diminution of pressure has the opposite effect to that obtained with sodium chloride and ferric hydrate as the permeability tends to decrease. The filter gradually becomes stained throughout and the permeability increases correspondingly.

Filtration of a Staining Crystalloid, Iodine in Potassium Iodide, through 11 per cent. Gelatine, under Varying Pressure.

Iodine was chosen as a crystalloidal substance having the property of staining gelatine, and potassium iodide was used as its solvent in water. The interest of the experiment centred chiefly in the relative behaviour of the two substances and the mutual influence they exert on each other during filtration under varying pressure.

Method.—A solution of 0·214 per cent. iodine in 1·54 per cent. potassium iodide was filtered through 11 per cent. gelatine in 1·54 per cent. potassium iodide. The content of the fractions of filtrate, etc., was determined by titration with sodium thiosulphate, using starch as an indicator for the iodine and with silver nitrate, using potassium chromate as indicator for the potassium iodide.

Examination for Iodine and Potassium Iodide.—Table II.

Original Fluid: 0·214 per cent. I_2 = (100) I_2. 1·54 per cent. KI = (100) KI.

Filtrates: The 1st c.c. contained a considerable percentage of iodine (5) and likewise of potassium iodide (68·8). On continued filtration at constant pressure the iodine concentration rose steadily to (25); the iodide rose slightly and then fell to (68·3). On suddenly diminishing the pressure from 100 to 15 atmospheres the iodine concentration diminished and the iodide increased. On raising to 100 atmospheres the iodine rose, whereas the iodide rose and then fell in concentration. Removing the pressure for 12 hours the few drops of liquid which percolated through contained no iodine and only (43) of

iodide. Re-establishing the pressure of 100 atmospheres the iodine content rapidly increased, whereas the iodide in the first few drops was abnormally high (93·5), but decreased in the second fraction of 6 c.c. to (86·0). Once more removing the pressure for three hours the iodine diminished to (1·0) and the iodide to (34·4), confirming the former result. The pressure was now slightly raised, viz., to 10 atmospheres, when the iodine became (25) and the iodide reached the highest value so far (94·6). A further rise in pressure to 100 atmospheres increased the iodine to (30) and diminished the iodide to (92·5). Variation between 100 and 50 atmospheres did not further affect the iodine content but did influence the iodide. Finally, a gradual fall in pressure to 25 atmospheres caused both iodine and iodide to diminish in concentration.

Residual Fluid: The concentration of iodine was only (30), whereas the iodide was (109·7).

Conclusions.—The gelatine absorbs a large amount of iodine, but is easily permeable to the same and the permeability rises and falls, as in the case of neutral red, with rising and falling pressure. The increasing concentration of iodine in the gelatine increases the power of the latter in retaining potassium iodide. On entirely removing the applied pressure the gelatine robs the liquid percolating through almost entirely of its iodine and the iodised gelatine abstracts about two-thirds of the iodide.

The filtrates, after a drop in the pressure, show an increased amount of iodide on recompression similar to the effect observed in the filtration of sodium chloride.

On the Influence of the Nature of the Solution and of Varying Pressure on the Rate of Filtration.

A few preliminary measurements of the rates of filtration of various solutions were made to elucidate some of the physical properties governing the action of the gelatine during filtration.

Method.—As a rough approximation the number of drops per minute falling from the nozzle of the filter case was taken as a measure of the rate of filtration. The rate was determined after the first 2 c.c. had passed, and again after about 50 c.c. in all had been collected.

Rates of Filtration.—Table III shows that distilled water, 0·8 per cent. sodium chloride and megatherium lysin pass 9 per cent. gelatine in the initial stage more rapidly than in the final under constant pressure. Solutions containing 0·214 per cent. iodine, with 1·54 per cent. potassium iodide, and 5 per cent. colloidal ferric hydrate pass 11 per cent. gelatine with similar variations in rate. For all these solutions the decrement in rate is practically

the same. On filtering horse serum in full strength, however, the decrement in rate was found to be abnormally large.

Table III.

100 atmos. 12° C.	NaCl.	KI, I.	NaCl lysin.	NaCl toluol.	NaCl Am.Alc.	NaCl HBū.	Dist. water.	Coll. $Fe(HO)_3$.	Serum.
Initial drops per minute	7	7	8	16	18	6	9	6	6
Final drops per minute	4	4	5	18	60	18	4	4	1

A solution of 0·8 per cent. saline containing toluol as a fine emulsion did not show any decrement.

On the other hand, 0·8 per cent. saline containing either amylic alcohol or butyric acid (4·8 per cent.) gave very distinct increments in the rate of filtration.

Table IV gives a comparison of the rates obtained for distilled water and 0·81 per cent. sodium chloride containing 4·8 per cent. of butyric acid. It is evident that distilled water filters more and more slowly, whereas the saline butyric acid passes more and more rapidly. The effect of removing the pressure for two minutes enables the distilled water on re-establishing the pressure to pass through at a higher rate, and if the pressure be removed for 10 minutes the gelatine regains completely its original rate of filtration.

Table IV.

Distilled water.				Sodium chloride and butyric acid.			
Pressure in atmos.	Time in minutes.	Drops per minute.	C.c. per minute.	Pressure in atmos.	Time in minutes.	Drops per minute.	C.c. per minute.
100	0	7	—	100	0	6	—
100	13	6	0·5	100	2	5	—
100	21	5	0·5	100	5	9·5	—
0	23	0	—	100	7	11	0·7
100	29	6·5	0·6	95	15	9·75	0·6
100	32	5	0·5	90	20	9	—
100	40	4·5	0·3	100	23	11	0·6
0	50	0	—	100	35	13	0·6
100	73	9	0·7	100	42	14	0·7
100	83	5·5	0·5	100	49	15	0·7
100	133	4·5	0·4	100	64	16	0·8
				100	71	18	0·9
				100	74	18	1·1

Conclusion.—Solutions of salts such as sodium chloride, or colloids such as ferric hydrate, will not appreciably modify the rate of filtration through gelatine unless in concentrated solution. Amylic alcohol and butyric acid accelerate the rate of filtration.

It seemed possible that this effect might be due to the condensation of amylic alcohol and butyric acid on the walls of the gelatine pores, and that the change in viscosity might account for the increased rate. This, however, does not seem probable, for a toluene emulsion did not markedly affect the rate of filtration, and as the viscosities of the toluene and amylic alcohol used were found to be 0·49 and 4·4 respectively when compared with distilled water as unity, one might conclude that the toluene would accelerate, and the amylic alcohol retard, the rate of filtration.

On the other hand, the action of amylic alcohol and butyric acid is consonant with the view that the surface forces between gelatine and saline are modified. The surface tension of saline against air diminishes with increasing concentration of both amylic alcohol and butyric acid. Both of these substances markedly influence the rates of sedimentation of solid suspensions through water,* and this fact seems to have a close connection with their influence on the rate at which water passes through a porous solid or jelly such as gelatine.

The effect of continued pressure on gelatine seems to be a tightening of the pores, which is nearly complete at 100 atmospheres in 40 minutes, and decompression apparently allows the pores to resume their original dimensions in about 10 minutes at atmospheric pressure.

On the Expression of Water and Gelatine from a Wet Filter.

In agreement with E. W. Reid, the filtrates were found to contain gelatine, but in variable amounts. Thus the first fraction of about 4 c.c. usually contained most, and after 20 c.c. had passed the gelatine was only present in traces. Rough estimations with tannic acid indicated about 0·5 per cent. gelatine in the first, and less than 0·1 per cent. in the fifth fraction on filtering distilled water. These contents of gelatine were not found to influence the titrations given above.

It has been hitherto assumed that the water in the pores of a wet filter is largely expressed before the liquid undergoing filtration appears in the filtrate undiluted by the same. For this reason Martin† discarded the first 10 c.c. of filtrate, and E. W. Reid‡ concluded that the concentration of even a second fraction of 25 c.c. might be considerably reduced by this dilution. It seems to me, however, that the effect of the water in the gelatine on the concen-

* *Cf.* Craw (1904). † *Loc. cit.* ‡ *Loc. cit.*

tration of the filtrate is neither so marked nor so long continued as has been imagined, for the following reasons. In the first place, the filtrates from saline through gelatine containing the same concentration of saline are at first only about half as concentrated as the original saline. Secondly, on decompression, and again filtering, the filtrate may contain a higher concentration of salt than the original saline. This seems conclusively to show that much of the dilution of the filtrate is due not to the water of the filter, but to the retention of the salt by the gelatine. Further, Reid found the quantity of water in freshly prepared filters to vary between 2 and 13 grammes—*i.e.*, presumably, the amount which can be removed by dry air. It is probable that only a small part of this water will be removed by the passage of a slow current of liquid through the gelatine, the remainder being retained by adsorption or inbibition forces, etc. In this connection an observation which was made with all the filtrations given in this paper may not be unimportant. It was found that the sum of the volumes of the filtrate fractions and residual fluid in any one filtration experiment was less than the volume of the original fluid introduced into the wetted apparatus. It is a well-known fact that water can be forced into gelatine by pressure, and as a large part of the gelatine in the pores of the filter, during filtration at 100 atmospheres, must be under considerable pressure, it seems reasonable to assume that part of the original water of the wet filter is more firmly bound, and that about 5 c.c. are imbibed partly from the water of the filter and partly from the fluid filtered. The rapid passage of iodine, neutral red, and colloidal ferric hydrate into the filtrate also point to a considerable percentage of the first fractions of filtrate being contributed by the fluid filtered. It seems, on the whole, as if the free water of the filter is almost wholly removed in the first fraction of 5 c.c. filtrate.

The Application of Mechanical and Adsorption Hypotheses to the Filtration Phenomena of Gelatine.

Mechanical Hypotheses.—The most obvious explanation of the retention of colloidal substances by the gelatine filter is that the colloidal molecule or grain is too large to pass through the pores. Martin* has advanced the view that the non-filtration may be due either to the size of the molecules or to some interaction between the colloidal membranes and the dissolved colloidal molecules. It has, however, been shown by E. W. Reid (1904), Gatin-Grazewska (1904), and others that proteids, glycogen, and other typically colloidal substances, exert no measurable osmotic pressure in solution, do not influence the freezing point of the aqueous medium, and in general diffuse

* *Loc. cit.*

very slowly, if at all. These facts lend much probability to Oswald's view that colloidal solutions may be regarded as very fine suspensions. As the molecular weights of these substances are as yet quite indefinite, it would seem more justifiable to speak of the colloidal "grain" than of the colloidal "molecule." Accepting this view of the nature of colloidal solutions, the action of the filter admits of an apparently satisfactory explanation on a purely mechanical basis.* During the filtration, for example, of colloidal ferric hydrate, Table II, the first fraction contained none of the colloid, and probably consisted chiefly of the water of the filter. In the succeeding fractions a little ferric hydrate passed through the larger pores, but these were speedily blocked by the particles. Under the pressure of 100 atmospheres the pores are probably compressed, and the effective passages are soon completely blocked by ferric hydrate. On suddenly diminishing the pressure the gelatine begins to return to its original condition, and the effective passages increase in number and diameter. The condensed ferric hydrate, no longer completely blocking the widened passages, is swept out of the gelatine by the oncoming fluid, and so gives a filtrate with an abnormally high concentration.

On the other hand, by gradually diminishing the pressure the ferric hydrate has time to fill the new and the gradually-widening pores, so that the filtrate contains only a trace of colloid. If the ferric hydrate be not expressed on recompression, it is conceivable that the compound filter of ferric hydrate and gelatine would be less affected as regards permeability by variation of pressure than the original gelatine. Much as this view has to recommend it, it does not seem to be the whole explanation, as it does not satisfactorily account for the phenomena observed with sodium chloride. It is highly improbable that sodium chloride is retained by a mechanical blocking of the passages, and that it is swept in high concentration into the filtrate, on decompression, because the condensed sodium chloride is no longer of the same dimensions as the widened passages. Recourse must, therefore, be had to some other view, which may considerably modify the explanation given for colloidal substances.

Adsorption Hypotheses.—Porous substances, powders, and fine suspensions of solids in aqueous media have the power of removing salts and other substances from solution. The action is attributed to forces of the same nature as those which give rise to adhesion and the wetting of a solid by a liquid, *e.g.*, of clean glass by water. The phenomenon usually called adsorption is common to all substances with highly developed surfaces, and

* *Cf.* also M. Traube (1866, 1867). Traube regards precipitation membranes as atomic and molecular sieves.

the intensity of the adsorption depends not only on the physical condition but also on the chemical nature of the active surface. Gelatine being, probably, a porous substance, presents a large surface to the enclosed fluid, and it seems highly probable that when this fluid contains crystalloids or colloids adsorption will take place. Thus, gelatine containing 0·8 per cent. saline apparently retains about one-half of its content in sodium chloride, and the retention of crystalloids and colloids on filtration through gelatine at constant pressure seems to admit of explanation on the basis of adsorption. The results obtained in the investigation of the adsorption phenomena of the sedimentation of silts through aqueous solutions may therefore be applied to what apparently is the converse of that process, viz., the passage of aqueous solutions through porous solids or powders, under pressure. This is rendered highly probable from the fact that the rate of sedimentation of a silt through saline is generally accelerated by the presence of butyric acid, amylic alcohol, and other substances influencing surface tension, and similarly the rate of filtration through the porous gelatine is accelerated by the same substances. An additional factor may, however, come into play in gelatine filtration. The effect of pressure on gelatine containing a solution of salt will be to cause the absorption of water. It seems permissible to assume, in view of Hardy's work on the structure of gelatine, that this additional water will be taken up by the web mass and will probably lead to a diminution in dimensions of the pores or web spaces. This view would account for the continued diminution in rate of filtration at constant pressure. The process should show some similarity to the passage of a solution into filter paper, where the water passes in more readily than the substance in solution.

A sudden diminution in pressure will lead to the rapid expulsion of the imbibed fluid, which will sweep the adsorbed matter into the widening pores. The gelatine itself will thus express part of this concentrated fluid, and at low pressures highly concentrated filtrates will be obtained. This will take place markedly when the adsorption is easily reversible, but the less reversible adsorptions of certain staining substances, *e.g.*, neutral red and iodine will not give this effect. Further compression and decompression would presumably lead to a more uniform distribution of the adsorbed substance throughout the gelatine, and as the filter is now also more saturated, the effect of variation of pressure on the concentration of the filtrate would be less marked.

Summary of Conclusions.

1. Wet gelatine filters are to be preferred to those which have been partially dried, as the former have more uniform rates of filtration, and dilution of the filtrate by the water of the gelatine can be largely eliminated.

2. Under constant pressure the gelatine of the filter absorbs water, and its porosity gradually decreases; on decompression this water is expressed, and the original porosity is rapidly regained.

3. Gelatine of a definite concentration apparently has a specific permeability for different crystalloids and colloids; the value is high but not complete for the crystalloids sodium chloride, potassium iodide, and butyric acid, and it is low, but not zero, for the colloids ferric hydrate, serum and soluble starch.

4. As filtration proceeds the crystalloids pass through in increasing concentration, whereas the colloids rapidly decrease to zero.

5. The simultaneous filtration of two substances may influence their specific permeabilities, thus butyric acid alters the permeability to sodium chloride, and iodine that of potassium iodide.

6. Variations in the gelatine influence the permeability, *e.g.*, formalised gelatine is less permeable to sodium chloride than ordinary gelatine, and 15 per cent. gelatine is less permeable to megatherium lysin than 7·5 per cent.

7. Variation in the pressure causes remarkable changes in permeability. A sudden diminution of pressure gives highly concentrated filtrates of both crystalloids and colloids, whereas a gradual diminution has practically no effect.

8. Substances which stain the gelatine, *e.g.*, neutral red and iodine, give filtrates with lower concentrations on diminishing the pressure.

9. The rate of filtration is accelerated by amylic alcohol and butyric acid, which accelerate the rate of sedimentation of silts in a similar way.

10. Part of the phenomena may be explicable on the mechanical view of a blocking of the gelatine pores, but chemical relations between the gelatine and substances filtered must be taken into consideration, and probably the most satisfactory view is that the action of gelatine on the solutions tested is essentially an adsorption phenomenon.

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Researches on Certain Problems of Plague Immunity.

THE object of the present communication is to record the results of certain researches on Plague Immunity which have been carried out in the Plague Department of the Lister Institute.

The chief problems dealt with in this contribution may be arranged as follows :—

1. Does the B. pestis produce a toxin capable of demonstration and suitable for experimental purposes ?
2. If such a toxin is obtainable, does its injection into the animal body result in the production of an antitoxin, and in how far does the ordinary anti-plague serum, which undoubtedly confers protection on such animals as rats and mice, owe its immunising property to an antitoxic action ?
3. By what mechanism does an anti-plague serum exercise its anti-infectious effect ?

It is unnecessary here to enter into all the details of the methods of preparing anti-plague serum, but for the sake of clearness in discussing certain of the questions connected with the above problems it seems desirable to give a brief outline of the subject.

As will be readily understood the most rigid precautions are necessary to obviate the risks of infection during the process of immunisation.

The ordinary procedure for obtaining an anti-microbial serum is employed. The animals first undergo a course of intramuscular, or intravenous, injections of killed cultures of the bacilli, till a certain degree of immunity is acquired. In the case of the plague bacillus this preliminary stage in the immunisation occupies a considerable period, extending from four to six months, or even longer, the slow absorption of the œdematous and indurated swellings which result from the subcutaneous and intramuscular inoculations being chiefly responsible for

the prolongation of the process. On the other hand, considerable risks of thrombosis, causing sudden death, are attached to the intravenous injection of killed cultures. This danger appears to be much greater with killed than with living cultures, and is probably due to the setting free by the heating to which the culture is subjected of a ferment or of nucleo-protein.

The further stage in the process of immunisation consists in giving the animal a series of intravenous injections of living virulent bacilli. The general experience has been that the injection of even a small quantity of living culture greatly increases the protective action of the serum.

Roux and Dujardin-Beaumetz at the Pasteur Institute Paris, and Calmette at the Lille Institute, prefer intravenous injections throughout, and use bacterial emulsions, prepared from agar cultures, for both the early and later stages, whereas Tavel Krumbein and Glücksmann at Berne use old broth cultures, killed by heating to 65-70° C. (Haffkine's Prophylactic Fluid), injected subcutaneously or intramuscularly in the early stages, and young broth cultures injected intravenously in the later stages of immunisation.

When "anti-plague serum" is referred to in this paper, a serum prepared by one or other of these methods is indicated. In certain of the following experiments the serum used was from animals which had been treated with heated or unheated filtrates only.

Brief Review of the Literature on Plague Toxin.

Yersin, Calmette and Borrel (1895) stated that the filtrates from broth cultures of the B. pestis have no toxic action on experimental animals, but that emulsions from agar of the bodies of the bacilli, which have been killed by heating to 58° C., cause the death of guinea-pigs and rabbits when injected subcutaneously or intraperitoneally.

Roux is stated by Metchnikoff (1897) to have obtained very powerful plague toxins by employing an organism, the virulence of which had been elevated by animal passage in collodion sacs. The filtrate from a bouillon culture, precipitated by ammonium sulphate, gave a toxin of which the lethal dose for a mouse was one-fourth of a milligramme, for a rabbit four centigrammes. The guinea-pig is the most

resistant of the usual laboratory animals. The toxin is destroyed by a temperature of 70° C.

Lustig and Galeotti (1897) obtained toxic substances by extraction with a one per cent. solution of caustic potash, and subsequent slight over-neutralisation with a one-half per cent. acetic acid solution. The resulting precipitate is highly toxic, and is employed as a vaccine.

The German Plague Commission (1899) points out that certain animal experiments indicate that the toxic action is not an important feature of the pathogenic effects produced by the plague bacillus. They refer to the condition observed in monkeys, in which a great part of the body is affected with oedema, and where the exudate swarms with bacilli, and yet in these animals strikingly few symptoms of toxaemia are displayed.

Notwithstanding this view of the Commission, it cannot be denied that toxaemia is a marked feature in many cases of human plague. In a considerable proportion of all cases the lesions remain localised in the affected lymphatic glands, no bacilli being present in the blood-stream, and yet these cases die with the symptoms of profound toxaemia. It is certain that the pathogenic effects of the plague bacillus are the result of toxic action.

The English Plague Commission (1898-99), in speaking of the subject of toxin, states, "We, for our part, have tested the effect which is exerted by heat upon the filtrate from a plague culture. It results from these experiments that the soluble toxins which are, under certain conditions, developed in the fluid nutrient medium in which the plague bacilli are grown, are apparently not affected by exposure to a temperature of even 100° C."

The Austrian Plague Commission (Albrecht and Ghon, 1900), obtained results in the main similar to Markl's, to be described directly. Markl's work was done in part with the Commission. They obtained toxic filtrates from broth cultures, the toxicity of which increased with the age of the culture up to a certain period, two to three months, after which no increase took place. The filtrates from cultures even only five days old were toxic, and the toxins differed only quantitatively from those obtained from older cultures.

The bacillary bodies, from cultures on agar, which had been killed by heating to a temperature of 55°-60° C., produced toxic effects similar to those observed with the filtrates.

The toxic filtrates were, in their experience, fairly stable, but 20 minutes in water at a temperature of 100° C completely destroyed the toxicity for mice.

By means of injections of the filtrates they were able to obtain evidence of active immunity against toxin, "Giftfestigkeit," and the animals which had acquired this showed also some increase of resistance to bacterial infection.

Markl (1898 and 1901) concluded, from a large number of experiments, that the plague bacillus, when grown in bouillon, under aerobic conditions, is capable of producing soluble toxins. The bacillus is grown in bouillon, in flat flasks, to allow a free supply of oxygen, since, under anaerobic conditions, no toxin formation takes place. Though toxin may be demonstrated in a very short period, even within 24 hours, two months at room temperature are required for the maximum toxicity of the cultures to be attained. The optimum temperature for toxin production is room temperature; 37° C., while admitting of toxin formation, is less favourable, a circumstance due partly to the injurious influence on the bacillus of the higher temperature, partly to its deleterious action on the already formed toxin. This susceptibility of the toxin to the action of heat is a marked feature; a temperature of 70° C., for 15 minutes completely destroys the toxicity of the filtrate for mice, whereas it still remains toxic for rats, rabbits and guinea-pigs. From this observation Markl argues that the filtrates contain two different, but related, toxins. The relationship is best shown by the fact that animals which have been injected with heated filtrates furnish an antitoxic serum which is capable of protecting mice against the unheated toxin.

Markl believes that, by injecting goats and horses, with toxin, an antitoxin may be obtained, and he suggests the advisability of employing an anti-plague serum, obtained by a combined method of immunisation with bacilli and with toxins.

He regards the toxin as a product of genuine bacillary secretion, and not merely as the result of the degeneration and death of the bacilli, with subsequent soaking-out of intracellular poisons. He bases his opinion on a number of observations:—First, the toxin is demonstrable at a very early period in the history of the culture, when the number of dead and degenerated bacilli is too small to account for the toxin present; second, the toxin production bears a certain relationship to the

virulence and habit of the culture. To obtain powerful toxins, the bacillus must have been recently isolated from the animal body. After a period of cultivation on artificial media, and especially if high temperatures have been employed, it loses its toxigenic property, which may, however, be recovered in part at least by fresh passage through the animal body.

Kolle (1903), in proceeding to study the production and nature of plague toxin, selected for his experiments such cultures as, from their behaviour in the animal body, might be expected to prove toxigenic on artificial media. He observed that, after a large number of passages through the rat, plague cultures showed a tendency to lose their property of multiplication in the blood, tissues, and organs of the animal body. The death of the animals resulted almost entirely from the toxins produced at the site of inoculation and in the neighbouring buboes. In other words, the cultures, before their frequent passage, tended to produce a plague septicaemia, after their passage a plague toxaemia. The toxaemic symptoms came on at an early period, when the multiplication of the bacilli in the rats was occurring solely at the site of inoculation. Kolle employed an alkaline bouillon.

Whatever the nature of the flask employed for the cultivation of the bacillus provision was made for an abundant supply of oxygen by having only a moderately thin layer of medium at the bottom of the vessel. Occasionally a little blood from the rat or rabbit was added to the bouillon, in which case the culture was observed to be very abundant. The cultures were grown some at 37° C., others at 35° C., others at 22° C., and still others in the ice-safe at from 6° - 10° C.

A number of methods were employed to obtain the toxin, apart from the bacilli. By the addition to the cultures of phenol and toluol, and subsequent centrifugalisation, a sterile supernatant fluid was obtained which was tested for toxicity. The Chamberland filter was also employed, the filtrate being tested for sterility, as in certain cases the plague bacilli have been found to pass the filter.

Kolle employed for his experiments rats of 180-200 grammes weight. In his experience the guinea-pig and rabbit are too resistant, while the mouse gives irregular results or proves too susceptible. Intraperitoneal injection was in every case employed.

In young cultures, of from 3 - 4 days, no toxin could be demon-

strated in the filtrates. On the other hand, if the bacillary bodies were present, the cultures had a markedly toxic action. From this it must be deduced that the toxin is bound to, or more probably contained within, the bacillary bodies. Such toxins have an incubation period of 6 - 8 hours, death usually occurring within 24 hours. The symptoms are those of collapse and giddiness, and point to the action of a brain and heart poison. The common effects of a bacterial disease are manifested in the spleen, bone-marrow and liver, viz., fatty degeneration and coagulative necrosis. The peritoneum is injected and may present small hæmorrhages, which may also occur in other organs. Kolle regards this as the primary specific plague toxin, since in the human subject similar effects are not infrequent. In animals which survive the acute toxic effects marasmus may occur.

In older cultures of from 7 - 10 days, where the growth has taken place at 37° C., an increase in the toxicity of the filtrates, and in the character of the resulting intoxication, can be determined, whereas the filtrates from cultures at lower temperature produce effects similar to those obtained from cultures 1 - 2 days old at 37° C. The older the culture, up to a certain point, the higher is the toxicity. The cultures at 30° - 37° C. reach their maximum toxicity in 3 - 4 weeks, whereas those at lower temperatures require 8 - 10 weeks.

The toxic action of the filtrates from the older cultures is characterised by a shortening of the incubation period. Symptoms of intoxication occur in 10 - 15 minutes, and in the case of the larger doses death may occur in 1 - 2 hours. The most prominent of the symptoms produced by these filtrates from old cultures are convulsions, from which the animals may recover. These effects are produced by non-specific substances of the nature of cadaverin and putrescin, which have also been demonstrated in old cultures of B. pyocyaneus, B. typhosus and B. cholerae. Associated with these substances, however, there are present in the old cultures specific toxins which may or may not be identical with those occurring in young cultures.

Kolle holds that the specific toxic substances originate not from a secretory process on the part of the bacillus, but are produced by the death of the bacilli with subsequent soaking-out of the intrabacillary toxin.

Such intra-cellular toxins never give rise to the formation of anti-

toxins. Kolle tested various anti-plague sera for antitoxic properties with negative results. He found that normal horse serum possessed the property in some degree, and that the slight toxin-neutralising properties of sera obtained by immunisation did not exceed the limits found in normal serum.

He attempted to immunise horses by intravenous injection of the primary toxin from young cultures, in others he employed the toxins from old cultures, but in neither case could any antitoxic property be demonstrated in the serum.

The serum and toxin were mixed, and injected peritoneally. Where 4 lethal doses of toxin were employed, 8 rats so treated all died. When 2 and 3 lethal doses of toxin were employed, a certain number of rats recovered, even when normal serum was used. Thus, out of 16 rats injected with a mixture of plague serum and toxin, 9 died; out of 8 injected with plague toxin and normal serum, only 5 died.

Kolle therefore concluded that the toxins found in plague cultures were intracellular toxins, and that consequently, in accordance with a general biological law, their injection into the animal body does not stimulate to antitoxin production. The question of the tenability of this so-called "general biological law" will be discussed later.

The three following investigations are grouped together because, in all, the toxic or immunising substance, is derived from the animal body.

Terni and Bandi (1900), proposed to employ, as an anti-plague vaccine, the peritoneal exudate obtained from guinea-pigs and rabbits which had been infected intraperitoneally, and killed during the death agony. The fluid, after being tested to ascertain that it was free from organisms other than the B. pestis, was incubated for 12 hours, and then submitted to a temperature of 50° - 52° C. for two hours. The fluid was brought to a suitable volume by means of a weakly alkaline normal salt solution, containing 0·5 % phenol. The authors claimed that good immunity was obtained, even in 4 to 5 days, and that the injection did not aggravate an already existing infection, but rather tended to prolong life. The blood of an individual who had received an injection of 1·5 to 2·5 c.c. showed, within 8 to 10 hours, a hindering influence on the development of the B. pestis.

Hueppe, F., and Kikuchi, Y. (1905), starting from the work of Bail, Kikuchi and Weil, propose to employ, as an immunising agent, the

peritoneal exudates obtained from guinea-pigs infected in the peritoneum with B. pestis. By means of two prophylactic injections of this fluid or "aggressin," they succeeded in conferring a high degree of immunity on mice, guinea-pigs, and rabbits. The injection of the substance was followed by a period of hypersensibility. The theory of the method and its relation to those of other workers will be discussed at a later period.

Klein (1905), while investigating the vitality of the B. pestis in certain organs, spleen and lung, and in the buboes of animals dead of plague, found that though all the bacilli had been destroyed by the process of drying over sulphuric acid, an emulsion of the organs, when injected into mice, was capable of causing the death of these animals within 20 hours.

All the phenomena of acute plague are exhibited, without, however, the B. pestis being present in the tissues. When the amount of emulsion injected was insufficient to cause death, the animals, after recovery from the local swelling and constitutional disturbance which resulted from the injection, were found, on being tested at a later period, to be refractory to infection, even when a virulent plague bacillus was injected.

Guinea-pigs, inoculated cutaneously with a bacillus of moderate virulence, develop a sub-acute form of plague, death occurring from the fourth to the ninth day. The lesions found are necrotic buboes, necrotic nodules in the spleen and liver, and particularly necrotic nodules and patches in the lungs. In sections of such organs the central parts of the necrotic nodules and patches are found to be crowded with bacilli, whereas the peripheral parts, consisting of debris, contain few, if any, bacilli. From this it may be inferred that the necrotic changes are due, not to bacilli themselves, but to their toxin.

Klein proposes to prepare a prophylactic substance from such material. The bubo, enlarged spleen, necrotic lung and liver of guinea-pigs which have died from sub-acute plague, are cut out, minced aseptically, and then spread out in thin layers on sterile glass dishes, and dried over sulphuric acid at 46° - 47° C. There is thus obtained "a material which not only can be very easily and rapidly prepared, but which is of a uniform and reliable efficacy, and in every way indeed superior to any of the other prophylactics." Klein calculates that a single large guinea-pig would yield 800 - 1000 human doses of the new prophylactic.

It is unnecessary here to go more minutely into the results obtained on animals, or the advantages claimed for the use of this material. From our present standpoint, however, the toxity of the substance is of interest.

(1) It kills a large percentage of mice within 20 - 24 hours in doses of 1 - 5 milligrammes.

(2) Prepared from acute cases, it kills 15 - 25 % of half-grown white rats in doses of 5 - 8 milligrammes; prepared from sub-acute cases, 10 - 12 milligrammes are required to produce a fatal effect.

(3) 20 milligrammes fail to kill a guinea-pig of 200 - 300 grammes weight.

Klein holds that the effects produced by the substance are due not merely to the bacilli contained in the material, but depend largely on the presence in the necrotic area of a "tissue toxin."

Besredka (1905 and 1906) has proposed a new method for the preparation of typhoid and plague endotoxins.

The growth from an agar culture of B. pestis is emulsified, heated to 60° C. for one hour, and then dried in vacuo. The lethal dose for the mouse of this dried substance, injected subcutaneously, is five decimilligrammes. To obtain the plague endotoxin in the soluble state, the dried bacilli are put in normal salt solution and horse serum in fixed quantities. The mixture is placed in the ice-safe overnight, and is centrifugalised on the following day. The clear supernatant fluid contains the endotoxin, and the deposit of a sticky consistence is formed of atoxic plague bacilli.

The soluble endotoxin is highly toxic for the mouse, *e.g.*, 0·02 centigramme of dried bacilli, to which 1 c.c. of physiological salt solution and 4 c.c. of horse serum have been added, corresponds to about 20 lethal doses of endotoxin.

The plague endotoxin keeps for months in the ice-safe. It is thermostable; heating to 55° C. for one hour leaves it intact; it is slightly attenuated by heating for one hour to 65° C., but it is not destroyed by heating to 57° C. for 15, or even 24 hours.

Its essential characteristic, according to Besredka, is that its action is neutralised by anti-plague serum.

Besredka also investigated the endotoxin of the typhoid bacillus, and

found that 32 lethal doses of the endotoxin are neutralised by 0·2 grammes of the serum of an immunised horse.

Similarly, he found that the serum of horses which had been injected intravenously, either with plague bacilli or with the soluble endotoxin, was capable of neutralising the plague endotoxin.

The Writer's Experiments on Plague Toxin.

The writer's experiments on the subject of Plague Toxin were directed chiefly to the study of the influence of certain factors on the toxicity of cultures of different races of the plague bacillus.

Technique used for obtaining the Toxin.—In certain cases the cultures were well shaken with toluol, the bacilli allowed to sediment, and the bacterial free supernatant fluid examined; but for the most part the filtrates of cultures were investigated. The filtration was carried out by means of a "tandem" filter, *i.e.*, the fluid was first passed through a Berkefeld, and afterwards through a Pasteur-Chamberland filter. The more porous Berkefeld filter removes the coarser particles, and this prevents the blocking of the Pasteur-Chamberland candle which, employed in this way, always gave a sterile filtrate. The loss of toxin is perhaps less by this method than if the Pasteur-Chamberland candle alone were employed, because in the latter case the blocking of the pores by the larger particles tends to make a very close filter. In every case it is desirable to test the sterility of the filtrates and centrifugalates.

Temperature.—The temperature most frequently employed for the growth of the cultures was what is generally accepted as the optimum temperature for the growth of the B. pestis, viz., about 30° C.

The Medium.—The medium employed was the ordinary bouillon such as is used in the preparation of diphtheria toxin. In most cases it was made neutral or very slightly alkaline to litmus, but, with the object of ascertaining whether toxin formation would be aided by the use of an alkaline broth, in certain cases the broth was prepared as for diphtheria toxin, 7 c.c. per litre of normal caustic soda being added after neutralisation with litmus as the indicator. The results obtained with the filtrates from two flasks of the same broth, in the one case neutral, in the other case alkaline, are shown in Tables I. and II. In this case, the neutral broth appeared to be the more favourable medium for toxin production.

TABLE I.—TOXIN 15.

Filtrate from a 2 month's growth on Neutral Broth.
Test on Mice of 20-25 grammes weight.
Subcutaneous injection.

Mouse.	Volume of Toxin.	Result.
1.	·05 c.c.	Lived.
2.	·1 "	Died 48 hours.
3.	·3 "	Died 24 hours.
4.	·5 "	Died 24 hours.
5.	1·0 "	Died 24 hours.

TABLE II.—TOXIN 16.

Filtrate from 2 month's growth of the same bacillus and on the same broth as Toxin 15. In this case the broth was made alkaline, 7 c.c. of normal caustic soda per litre being added after the neutral point to litmus was reached.

Test on Mice of 20-25 grammes weight.
Subcutaneous injection.

Mouse.	Volume of Toxin.	Result.
1.	·05 c.c.	Lived.
2.	·1 "	Lived.
3.	·3 "	Lived.
4.	·5 "	Died 48 hours.
5.	1·0 "	Died 24 hours.

It was observed that the addition to the broth of 1 : 4 to 1 : 8 of normal horse serum heated to 65° C., gave a very abundant growth, in some cases with pellicle formation, but so far as this was investigated no increase in toxin formation could be demonstrated.

The cultures were grown for the most part in large flat bottles each containing 1 to 1½ litres, but Erlenmeyer's flasks, with a thin layer of medium in the flask, were also employed. Previous experience had

shown that under these conditions the supply of oxygen is sufficient to satisfy an organism so exigeant in this direction as B. diphtheriæ.

Appearance and Age of the Culture.—The cultures vary greatly in the appearances they present. In certain cases the growth begins as minute flocculi in the bouillon, the surrounding fluid remaining clear. Other cultures show an almost uniform turbidity. To certain of the flasks cocoa butter or butter was added, and in these good stalactite formation was observed. No connection between the type of growth and the toxicity could be determined. The culture, as it increases in age, becomes very alkaline, and in two months there is usually a large deposit containing bacterial debris and masses of crystals of triple phosphates.

One change is of considerable significance in relation to toxin formation. A platinum loopful from a culture a few days old, planted on an agar plate, gives an abundant growth, whereas, from a flask two months old, little or no growth may be obtained unless a larger quantity of material be employed for inoculation. In the two months' old culture, almost all the bacilli present have lost the usual morphological characters of the plague bacillus, the deposit in the flask consisting almost entirely of bizarre involution forms or mere detritus.

The Period of Maximum Toxicity.—Some preliminary tests were made on the toxicity of the filtrates from cultures 10 days and two months old in bouillon of the same brew. Such a test shown in Tables III. and IV. indicates that at 10 days, if toxin is present, its concentration is much less than at two months.

TABLE III.—Toxin 7.

Filtrate from 10 day's growth on Neutral Broth.
Test on Mice 15-20 grammes weight.
Subcutaneous injection.

Mouse.	Volume of Toxin.	Result.
1.	·05 c.c.	Lived.
2.	·1 "	Lived.
3.	·5 "	Lived.
4.	1·0 "	Lived.

TABLE IV.—Toxin 13.

Filtrate from 2 month's growth on same Neutral Broth as No. 7.

Test on Mice 15-20 grammes weight.

Subcutaneous injection.

Mouse.	Volume of Toxin.	Result.
1.	·05 c.c.	Lived.
2.	·1 "	Died 48 hours.
3.	·3 "	Died 48 hours.
4.	·5 "	Died 24 hours.
5.	1·0 "	Died 24 hours.

It thus appears that the maximum toxicity of the culture is synchronous with the period when most of the organisms have undergone degenerative changes or have died ; it seems probable, therefore, that the toxic substances have been set free as the result of a process of autolysis. Reference will be made to this in discussing the question of toxins. Most of the filtrates tested were from cultures 8 to 10 weeks old.

Great variations were observed in the toxicity of the filtrates from different brews of bouillon made by the same methods and with the same proportions of the components. Similar inexplicable variations are well known in the preparation of diphtheria and tetanus toxin.

It would be impossible here to give the protocols of all the tests carried out in ascertaining the toxicity of different filtrates, but those recorded are typical of the results obtained in the case of the more toxic filtrates. A number examined were found to be much less toxic than Toxin No. 10. As this toxin was employed for a test toxin in testing the sera, the protocols of tests on mice and rats are given (Tables V., VI., VII. and VIII). It will be noted that there is a certain amount of irregularity in the results on mice, which makes the determination of the minimal lethal dose a matter of some difficulty.

TABLE V.—Toxin 10.

Filtrate from culture on Neutral Bouillon 10 weeks old.
Tested on Mice of 20-25 grammes weight.
Subcutaneous injection.

First Test.

Mouse.	Volume of Toxin.	Result.
1.	·01 c.c.	Lived.
2.	·02 "	Ill—recovered.
3.	·03 "	Ill—recovered.
4.	·04 "	Died 8 days.
5.	·05 "	Ill—recovered.
6.	·1 "	Died 24 hours.
7.	·2 "	Died 24 hours.
8.	·5 "	Died 24 hours.
9.	·5 "	Died 24 hours.

TABLE VI.—Toxin 10.

14 days after previous Test on Mice of 15 grammes weight.
Subcutaneous injection.

Second Test.

Mouse.	Volume of Toxin.	Result.
1.	·05 c.c.	Died 24 hours.
2.	·1 "	Died 24 hours.
3.	·2 "	Died 24 hours.
4.	·3 "	Died 48 hours.
5.	·4 "	Died 24 hours.
6.	·5 "	Died 24 hours.
7.	·6 "	Died 24 hours.

TABLE VII.—Toxin 10.

14 days after previous Test on Mice of 15 grammes weight.
Intraperitoneal injection.

Second Test (continued).

Mouse.	Volume of Toxin.	Result.
1.	·02 c.c.	Died 24 hours.
2.	·05 "	Died 24 hours.
3.	·1 "	Died 24 hours.

TABLE VIII.—Toxin 10.

Testing Toxicity on Rats.
Subcutaneous injection.

Rat.	Weight in grammes.	Volume of Toxin.	Result.
1.	125	·1 c.c.	Died 3 days.
2.	125	·5 "	Died 24 hours.
3.	150	1·0 "	Died 24 hours.
4.	155	2·0 "	Died 48 hours.

It is interesting to note that rats and mice offer much less resistance to the action of plague toxin than do rabbits and guinea-pigs. That the latter animals have the more powerful defence is shown by the experimental results given in Tables IX. and X.

TABLE IX.—Toxin 10.

Testing Toxicity on Guinea-pigs.
Subcutaneous injection.

Guinea-pig.	Weight in grammes.	Volume of Toxin.	Result.
1.	240	·1 c.c.	Unaffected—lived.
2.	240	·5 "	" "
3.	260	1·0 "	" "
4.	250	2·0 "	" "
5.	250	3·0 "	" "
6.	260	4·0 "	" "

TABLE X.—Toxin 10.

Testing Toxicity on Rabbits.
Subcutaneous injection.

Rabbit.	Weight in grammes.	Volume of Toxin.	Result.
1.	550	·05 c.c.	Unaffected—lived.
2.	500	·1 "	" "
3.	520	·5 "	" "
4.	526	1·0 "	" "
5.	950	2·0 "	" "
6.	760	3·0 "	" "
7.	800	4·0 "	" "

All guinea-pigs and rabbits alive at the end of one month.

In regard to the stability of the toxin, the writer's experience was in agreement with Markl's. The toxins were found to be rather unstable, especially at ordinary hot weather temperatures as, for example, when Toxin 10 was tested two months after the test recorded in Tables V., VI., VII. and VIII., it was found to have so far deteriorated that the minimal

lethal dose had risen from the 0·1 c.c. of the Table to 0·8 c.c. Higher temperatures, as might be expected, cause similar deterioration in a much shorter time. The effect of exposing the toxin to a temperature of 70° C. is shown below:—

TABLE XI.

Showing influence of heat to 70° C. on Toxin No. 10.

Mouse.	Weight in grammes.	Volume of Toxin.	Result.
1.	20	1 c.c.	Dead in 24 hours.
2.	15	1 "	Dead in 6 hours.
3.	20	1 "	Dead in 8 hours.
		Toxin heated to 70° C. for 30 minutes.	
4.	15	1 c.c.	Lived.
5.	15	1 c.c.	Lived.
6.	20	1 c.c.	Lived.

Where irregularities occur, such as are frequent in the testing of plague toxin, it is extremely difficult to trace the influence of the virulence of the race of bacillus on the toxicity. It was found impossible to establish any definite relation between virulence and toxicity, but a strong impression was formed that as a culture diminished in virulence the filtrate obtained from it became less toxic.

Incubation Period.—The incubation period lasts from 6 to 24 hours according to amount of toxin injected. In certain cases, where very old cultures are used, convulsions occur almost immediately after the injection, but these are probably due to the presence of cadaverin, as has been suggested by Kolle. These frequently pass off in a short time to be succeeded by the symptoms of toxic action.

Effects and Symptoms resulting from the Injection of Toxin.—If the injection is subcutaneous, within 24 hours a soft œdematous swelling, often very extensive, is observed in the vicinity of the site of inoculation.

In acute cases, after the period of incubation, the animal hunches itself up in a corner of the cage, refuses to eat, and, when disturbed, moves in an inco-ordinate manner, as if suffering from alcoholic intoxication. Convulsions are frequent just before death, which appears to be due to collapse.

In chronic cases the early symptoms are less acute, the animal gradually loses flesh till a profound state of marasmus may result. In some of the rats suffering from chronic toxæmia following the injection of toxic filtrates, we have observed a form of paralysis resembling diphtheria paralysis in the guinea-pig. The hind limbs are first affected, then the fore limbs, and ultimately the respiratory muscles may be involved. These animals are often greatly emaciated. Convulsions frequently occur immediately before death.

Post-mortem Changes observed in Animals dying of Toxæmia.—In the animals which die of acute toxæmia, say within 24 hours after subcutaneous injection of a toxin, the most marked feature is œdema round the site of inoculation. This may be very extensive, affecting the whole abdominal wall, and small hæmorrhages are sometimes present. The neighbouring lymphatic glands may be enlarged. Hæmorrhages are sometimes present in the viscera, especially where the injection has been intraperitoneal, in which case the peritoneum and the mucous membrane of the alimentary canal are injected or hæmorrhagic.

In the animals which survive several days or weeks, wasting of the musculature is a marked feature. Fatty degeneration of the liver is a frequent lesion, and necrotic areas are present in a few of the cases. Where death occurs at a late period the spleen may be enlarged.

The Question of a Plague Antitoxin.

With the view of ascertaining whether the injection of toxic filtrates would stimulate the animal body to antitoxin production, two horses were specially employed for immunisation with the filtrates of cultures. In the case of one, the horse "R" filtrates from bouillon cultures, which had been heated to 70° C. for one hour, were alone used. This animal, in the three months preceding the removal of the blood, the serum from which was tested, received injections amounting to 1080 c.c. of filtrates.

The second animal, after a course of injection of heated filtrates, was further treated with injections of filtrates of unheated cultures. In the three months preceding the bleeding from which the serum tested was obtained, nearly two litres of unheated toxin were injected in doses of from 60 to 300 c.c. Among other toxins, nearly a litre of Toxin No. 10, the tests of which have been recorded, was employed in the immunisation.

As already mentioned, the other horses whose sera were tested were immunised by the ordinary methods. The case of the horse "Pr." requires some comment. This animal had been immunised by the Berne method, and had received large injections up to 300 c.c. at one dose of concentrated Haffkine's Prophylactic Fluid. This was followed, as is usual, by a number of intravenous injections of living broth culture. On reviewing the treatment which this animal had received before the removal of the sample of serum tested, it was noted that within the six weeks of treatment preceding the interval which always intervenes between the last injection and the venesection, the animal received the following injections:—One of 40 c.c. and another of 90 c.c. of broth cultures four days old, one of 100 c.c. two days old, and one of 40 c.c. six days old. In regard to this, it is to be noted that these injections were given rather more frequently than usual, and that 180 c.c. of the material was 4 to 6 days old when at least traces of toxin might be expected to be present.

It is well known that out of a number of horses immunised against diphtheria and tetanus toxins, only a certain number of these respond freely and quickly to the stimulus of toxin injections, and are capable of producing antitoxins of the highest potency. It is probable that this variability among the individuals of the same species, in the response to stimulation by toxin, may extend to other forms of immunisation. It is quite conceivable that the dosage mentioned may have in this case been a sufficient stimulus to excite the mechanism necessary for antitoxin production in a highly responsive organism.

To ascertain whether the injection of heated filtrates had given rise to antitoxin production, the following preliminary test was carried out with the serum of horse "R," and controlled with the serum of normal horse "Z," the test toxin employed being Toxin No. 10. The toxin and serum were mixed and kept one hour at 35° C. It will be seen in Table XII. that the evidence is in favour of antitoxin production.

TABLE XII.

TEST OF SERUM OF HORSE "R."

Test Toxin No. 10. Toxin and Serum mixed and placed 1 hour at 35° C.

Rat.	Weight in grammes.	Volume of Toxin.	Volume of Serum.	Result.
1.	100	2 c.c.	1 c.c.	Died in 8 days.
2.	100	2 "	1 "	Lived.
3.	110	2 "	1 "	Lived.
CONTROL:—Serum of Normal Horse "Z."				
4.	100	2 c.c.	1 c.c.	Dead in 24 hours.
5.	100	2 "	1 "	Dead in 24 hours.
6.	110	2 "	1 "	Dead in 24 hours.

A test was carried out on one day with the serum of a number of horses. It will be seen from the Table XIII. that the only serum which showed marked antitoxic properties was the serum of horse "M," which had been immunised with toxins only.

TABLE XIII.

SERUM OF SEVERAL HORSES VARIOUSLY IMMUNISED.

Test Toxin No. 10; tests all carried out on one day.

Toxin and Serum mixed and kept 1 hour at 35° C

	Rat No.	Weight in grammes.	Vol. of Toxin.	Vol. of Serum.	Result.
Horse "M."	1.	95	2 c.c.	1·0 c.c.	Lived.
	2.	100	"	0·1 "	Lived.
	3.	100	"	0·02 "	Lived.
Horse "R.D."	4.	100	2 c.c.	1·0 c.c.	Lived.
	5.	90	"	0·1 "	Died 48 hours.
	6.	90	"	0·02 "	Died 24 hours.
Horse "M.B."	7.	90	2 c.c.	1·0 c.c.	Lived.
	8.	90	"	0·1 "	Died 48 hours.
	9.	90	"	0·02 "	Died 48 hours
Horse "P.N."	10.	100	2 c.c.	1·0 c.c.	Died 48 hours.
	11.	95	"	0·1 "	Died 48 hours.
	12.	95	"	0·02 "	Died 24 hours

The test on the serum of "M." was repeated and controlled with the normal horse "N. H.," see Table XIV. The same Test Toxin No. 10 was used, and the toxin and serum were mixed and kept two hours at 35° C.

TABLE XIV.

TEST ON SERUM OF "M." REPEATED.

Serum of Normal Horse "N. H."

Rat No.	Weight in grammes.	Volume of Serum.	Volume of Toxin.	Result.
1.	100	1 c.c.	2 c.c.	Died 24 hours.
2.	90	0·5	2 "	Died 48 hours.
3.	95	0·1	2 "	Died 48 hours.
Serum of Horse "M."				
4.	100	1 c.c.	2 c.c.	Lived.
5.	100	0·1	2 "	Lived.
6.	95	0·02	2 "	Lived.
7.	90	0·01	2 "	Died 24 hours.

Reference has been made to the serum of the Horse "Pr." The results of a number of tests relating to the serum of this horse are shown in the accompanying Tables :—XV., XVI., XVII. and XVIII.

TABLE XV.

TESTING SERUM OF "Pr." (6. IV).

Mice of 15-20 grammes weight.

Toxin (Subcutaneously at least = 4 lethal doses; intraperitoneally = 10 lethal doses).

Serum and Toxin mixed and placed 1 hour in the incubator at 37° C.

Mixture injected subcutaneously.

Mouse.	Volume of Toxin.	Volume of Serum.	Result.
1.	·2 c.c.	·1 c.c.	Lived.
2.	·2 "	·1 "	Lived.
3.	·2 "	·2 "	Lived.
4.	·2 "	·2 "	Lived.
Mixture injected intraperitoneally.			
5.	·2 "	·1 "	Lived.
6.	·2 "	·1 "	Lived.
7.	·2 "	·2 "	Died in 48 hours.
8.	·2 "	·2 "	Lived.
CONTROLS :—Same quantities of Toxin; serum of normal Horse "F."			
Subcutaneous.			
9.	·2 c.c.	·2 c.c.	Died 24 hours.
10.	·2 "	·2 "	Died 16 hours.
Intraperitoneal.			
11.	·2 "	·2 "	Died 24 hours.
12.	·2 "	·2 "	Died in 10 hours.

TABLE XVI.

TESTING SERUM OF "Pr." (27, IV.) ON RATS.

Test Dose about 10 M. L. D.

Toxin and Serum mixed and kept 2 hours at 37° C., then injected subcutaneously.

Mouse.	Weight in grammes.	Volume of Toxin.	Volume of Serum.	Result.
1.	120	1·0 c.c.	·01 c.c.	Died 24 hours.
2.	120	"	·02 "	Lived.
3.	115	"	·05 "	Lived.
4.	115	"	·1 "	Lived.
5.	115	"	·3 "	Lived.
6.	110	"	·5 "	Lived.
7.	105	"	1·0 "	Lived.
8.	100	"	1·0 "	Died in 27 days.
CONTROLS:—Done with Serum of Normal Horse "F."				
9.	120	1 c.c.	2 c.c.	Died 3 days.
10.	105	"	2 "	Died 3 days.
11.	110	"	1 "	Died 24 hours.
12.	120	"	1 "	Very ill; recovered.*

* The rats frequently bite the site of inoculation, and in certain cases, as the result of this, leakage may take place.

TABLE XVII.

TESTING SERUM OF "Pr." ON MICE.

The Serum injected subcutaneously at one side of the body, and 24 hours later the Toxin at the other side.

Test Toxin No. 10 about 4 to 6 M. L. D.

Only small mice available.

Mouse.	Weight in grammes.	Volume of Serum.	Volume of Toxin.	Result.
1.	8	·6 c.c.	·3 c.c.	Lived.
2.	10	·6 "	·3 "	Lived.
3.	10	·6 "	·3 "	Lived.
4.	10	·6 "	·4 "	Lived.
5.	10	·6 "	·4 "	Lived.
6.	10	·6 "	·4 "	Lived.
CONTROLS:—With Serum of "B." (old Diphtheria Horse).				
7.	8	·6 c.c.	·3 c.c.	Very ill; recovered.
8.	10	·6 "	·3 "	Died 4 days.
9.	10	·6 "	·3 "	Died 7 days.
10.	10	·6 "	·4 "	Died 48 hours.
11.	10	·6 "	·4 "	Died 48 hours.
12.	15	·6 "	·4 "	Very ill; died 12 days.

TABLE XVIII.

TESTING SERUM OF "Pr." ON RATS (27, IV.).

Toxin No. 10.

Serum injected on one side of body; Toxin (at least 10 M. L. D.) 24 hours later on the other side.

Rat.	Weight in grammes.	Both subcutaneous at opposite sides of abdomen.		Result.
		Serum.	Toxin, 24 hours after Serum.	
1.	95	1 c.c.	1·5 c.c.	No symptoms.
2.	95	"	"	No symptoms.
3.	100	"	"	No symptoms.
4.	105	"	"	No symptoms.
5.	115	"	"	No symptoms.
6.	120	"	"	No symptoms.
CONTROLS:—With Normal Serum of Horse "Z."				
7.	95	1 c.c.	1·5 c.c.	Died 24 hours.
8.	100	"	"	Died 48 hours.
9.	100	"	"	Died 24 hours.
10.	110	"	"	Died 48 hours.
11.	115	"	"	Ill; recovered.
12.	120	"	"	Died 48 hours.

These tests appear to show :—

1. That the serum is capable of neutralising the toxin when mixed *in vitro*, as tested by subsequent subcutaneous or intraperitoneal injection into rats and mice.
2. That the serum injected into rats or mice, 24 hours before the toxin, is capable of conferring protection on these animals.
3. In the case of rats, 0·02 c.c. of serum, when mixed with the toxin, neutralises the toxic action of about 10 M. L. D., and 1 c.c. of serum protects against at least 10 M. L. D. injected at another part of the body 24 hours after the serum.

Kolle says that the serum of the normal horse has considerable antitoxic properties, but, as already stated, he was unable to obtain an increase beyond what he regarded as within the limits of normal variation.

If the serum of an animal normally contains antitoxin, we might anticipate on the basis of Ehrlich's theory that it would be possible by stimulating the organism to obtain an increase in the production of the antitoxin. In our experiments, for purposes of control, the sera of four normal horses were employed, but, in addition, the serum of five horses immunised by the ordinary method of obtaining anti-plague serum showed little or no increase in antitoxic power, and these may, therefore, be classed among the controls.

The evidence from these experiments seemed to be in favour of the possibility of obtaining a serum with antitoxic properties in excess of those found in normal serum.

Relation of Neutralisation of Toxin to Precipitin Reaction.—Certain experiments were carried out with the view of ascertaining whether the occurrence of the precipitin reaction of Kraus might not play some part in the plague toxin-antitoxin reaction.

The serum of certain of the horses gave a well marked precipitation with the Test Toxin No. 10. The serum of the horse "R D." gave 3 to 4 times the precipitum given by the serum of the horse "M." In the previous tests we have seen that whereas 1 c.c. of the serum of "R D." was required to neutralise 10 M. L. D. of Toxin 10, 0·02 c.c. of the serum of "M." produced the same effect. There is, therefore, no direct proportionality between the two properties of the serum.

The Protective Action of Anti-Plague Serum.—Though much sceptical criticism has been directed against anti-plague serum, it has been admitted by everyone who has seriously approached the subject that, however disappointing the results of its use on the human subject may be, it manifests definite protective properties when tested on animals. A typical test showing the effect on rats is shown in the annexed Table XIX.

It will be noted that death occurred in the case of the animals which received the largest dose of serum. The writer has observed similar results in the case of anti-streptococcic serum. Tavel, Krumbein, and Glücksmann also record similar results, and attribute them to the well-known phenomena of "diversion of the complement" easily demonstrated in test tube experiments on bacteriolysis.

A common experience is to find that animals treated with serum survive till about the tenth day, and then succumb to the disease. This is probably due to the fact that the immune serum has by that time been metabolised and execreted, and that some of the bacilli which have not been destroyed are able to re-infect the animal.

We have already seen that the antitoxic properties of the serum of the horse "MB." are not of a high order, and probably do not exceed the limits found in normal serum.

A number of tests were carried out to determine whether the serum possessed bacteriolytic properties. The methods employed and the results obtained are dealt with in the following section.

Testing Plague Serum for Bacteriolytic Properties.

A number of methods were employed to ascertain whether bacteriolysis could be demonstrated in the case of anti-plague serum. Neisser's (1904) technique was found to give the most consistent and accurate results.

Small test tubes of about 10 × 1·3 c.m. are employed. Suitable dilutions of the immune serum are prepared in normal salt solutions, say, 1 : 10, 1 : 100, 1 : 1000, etc., and the quantity of serum to be tested is pipetted into each test tube of a series, and to this 0·5 c.c. of fresh normal serum is added to supply complement. Three drops of bouillon are introduced into the mixture, and the volume in each tube is brought up to 2 c.c. A measured quantity of a suitable dilution of the culture is

TABLE XIX.

TESTING SERUM OF HORSE "MB."

Rat No.	Weight.	Serum 24 hrs. before toxin.	Infection.	Result and Remarks.										
				Days 1	2	3	4	5	6	7	8	9	10	Survivors killed on 15th day after inoculation.
1.	215	·01	Shaving cutaneous			+								
2.	175	·05	"				+							
3.	195	·1	"						+					
4.	110	·3	"											Lived.
5.	120	·5	"											Lived.
6.	210	1·0	"											Lived.
7.	190	2·0	"											Lived.
8.	145	3·0	"											Lived; plague pneumonia.
9.	220	4·0	"											Lived.
10.	120	10·0	"							+				
	Controls.													
1.	240	0	"			+								
2.	240	0	"					+						
3.	300	0	"			+								
4.	200	0	"			+								
5.	225	0	"				+							

Note.—"+" indicates death.

then introduced into each tube. The tubes are placed in the incubator at 37° C., and are examined at stated intervals, say in 3, 16, 24-48 hours, the same volume from each tube being pipetted on to an agar tube or Petri dish. The absence or diminution in the number of colonies on these tubes or plates after their incubation is, with certain reservations, regarded as the indication of bacteriolysis having taken place. The condition of the small test tubes, after their incubation for 24-48 hours, affords valuable information, as will be exemplified in the following test:

Test of the Serum of Horse "RD."—The arrangement in this experiment was as above. The bacterial dilution was prepared by emulsifying a 2 mgm. öse from a 24 hours' culture on agar of a highly virulent plague bacillus in 10 c.c. of normal salt solution (·85%). The bacilli were well distributed by shaking, and a 2 mgm. loopful of this dilution was introduced into each of the tubes containing the immune serum, fresh horse serum and broth. The agar tube planted at once from the control tube, which contained salt solution and bouillon but no serum, gave a fair number of colonies, which became confluent over the water of condensation. A measured quantity from each tube was planted on agar, after 3 and after 16 hours. The details and result of the experiment are shown in the Table XX.

TABLE XX.

Volume of Anti-plague Serum, Horse "R."	Volume of complemental normal horse serum (16 hours old).	Condition in agar tubes, after 24 hours at 37° C.	Condition in tube containing immune serum and complement after 48 hours.
1·0 c.c.	0·5 c.c.	0	Growth in agglutinated colonies through the fluid.
·3 "	"	0	" "
·1 "	"	0	" "
·03 "	"	0	" "
·01 "	"	0	" "
·003 "	"	Colonies; few.	" "
·001 "	"	Colonies; few.	" "
·0003 "	"	Colonies; many.	Homogeneous growth.
·0001 "	"	Colonies; many.	" "
0	"	Uniform growth.	" "

The experiment shows, therefore, that while there was an apparent inhibition of growth, probably largely due to the agglutinating power of the serum which was 1 : 300 with a virulent culture, in no single tube had complete destruction of the bacilli taken place, since growth occurred in every one of the tubes containing the serum mixture.

A number of similar experiments, some of them carried out with the immune horse serum so fresh that the addition of complement was unnecessary, gave perfectly consistent results, *e.g.*, the fresh serum of horse " M B." was tested as above with a bacillus of low virulence, in full strength and in dilutions of 1-10, and 1-100, and 1-1000, and growth took place in all the tubes.

No matter what method was adopted, it was found impossible to obtain evidence of the serum possessing any bacteriolytic property, even when bacilli of low virulence were used.

Discussion as to the Nature of the Mechanism by which Anti-Plague Serum Exercises its Anti-Infectious Effect.

In considering the properties of anti-plague serum we have seen that an antitoxic action can play little part in the protective influence exerted by this serum. It is conceivable that the plague bacillus produces and throws off a large number of toxic particles or receptors under the stimulus of the antagonistic influences which it encounters when introduced into the animal body. If this be the case, it seems reasonable to assume that the bacilli, even when grown in artificial media, possess, though in smaller numbers, those toxic particles which are liable to be set free, especially on the death or during the disintegration of the microbes.

In dealing with the literature of plague toxin reference has been made to several methods of immunisation which are based on the common assumption that certain toxic or immunising substances are produced in a concentrated form in the animal body from which they are recoverable. Thus Terni and Bandi, Hueppe and Kikuchi used peritoneal exudates, and Klein the necrotic areas resulting from plague infection in different organs, as their immunising agent.

The first work in this direction was done by Denys and Van de Velde (1895) who showed that a substance "leucocidine," highly toxic for leucocytes, could be obtained in the pleural exudates resulting from the injection of staphylococci into the pleural cavity of the rabbit, and that the serum of rabbits immunised against staphylococci contained a substance "anti-leucocidine," capable of neutralising the toxic action of leucocidine. The leucocidine could be demonstrated to be present in cultures though not so abundantly as in the exudates.

The whole subject of the toxic and immunising action of the body fluids of infected animals has been re-opened by the investigations of Bail and his co-workers. This work embraces a theory of the method of attack on the animal body by certain groups of bacilli, and of the nature of the defence by means of which immunity is attained. Bail's views may be briefly stated as follows:—Certain organisms obtain a footing in the animal body by means of substances which they throw off, and to which Bail gives the name of "aggressins." These are demonstrable in the bacterial-free body-fluids, exudates, etc., of the infected animal.

The aggressive property of such fluids can be shown in the following manner. A non-lethal dose of the bacillus becomes lethal when mixed with its specific aggressin. The presence of aggressin, along with a lethal dose, causes the occurrence of a disease of a severer and more acute type, and one in which the pathological lesions are of a more pronounced character.

It is, in the case of the strictly parasitic bacilli, such as anthrax, fowl-cholera and plague, where a single virulent organism is probably sufficient to cause infection, that the aggressin action is most marked. The living bacillus can, without losing its vitality, secrete or throw off particles which prevent its destruction by phagocytes, and enable it, at the same time, to multiply and produce its pathogenic effects.

The immunity obtained by the injection into the animal body of natural aggressins is attributed by Bail to the production of anti-aggressins.

Wassermann and Citron (1905) hold, that aggressins can be obtained outside the animal body by shaking bacteria in serum or in distilled water. The fluids obtained by these means, after removal of the living germs, are capable of aiding the infective process. Their view is, that the aggressins, whether obtained from the animal body or by this method,

act by combining with the lysin of the serum, and thus interfere with the bacteriolytic process.

Such artificial aggressins must be regarded as closely related to certain bacterial toxins. One need only recall here, that the method employed by Wassermann and Citron is practically identical with Besredka's method for obtaining plague toxin.

The powerful immunising action of the natural aggressins, compared with the weak immunising action of these artificial aggressins, is used by Bail as an argument in favour of the essential difference in the nature of the two groups of bodies.

It is impossible here to enter into a full discussion of the relation of the aggressins to the "free receptors" of Neisser and Shiga (1903), and to the substance, demonstrated by Pfeiffer and Friedberger (1905), in serum, after its treatment with emulsions of typhoid and cholera organisms.

The intimate relation which these subjects bear to plague immunity is emphasised by certain work carried out in the Laboratory here, by Captain Douglas, I.M.S. (1906), recorded in a paper read at the Pathological Society of London, in which he showed that, contrary to the view taken by the German and English Plague Commissions, the supernatant fluid of Haffkine's Prophylactic Fluid has powerful immunising properties, as had been previously maintained by Haffkine. Where the prophylactic fluid was prepared by emulsifying the bacillus from agar in normal salt solution, the supernatant fluid, in this case also, was demonstrated to possess an immunising property. The writer has shown that the injection of the supernatant fluid in the horse gives rise to the production of a small amount of antitoxin. It is difficult to reconcile with these results of Douglas the statement made by Bail and Weil (1906), that attempts to immunise with watery extracts against the true parasites prove absolutely futile, since the B. pestis has been classified by them in this group.

Neisser and Shiga in the case of the dysentery bacillus of Shiga and the typhoid bacillus, obtained analogous results which they attributed to the presence of free receptors in the filtrates from the autolysed bacilli. In that case bacteriolytic sera were obtained ; in the case of plague we have shown that no matter whether the two fluids employed by Douglas and the writer or the living bacillus are used in the treatment of the

animals, the serum obtained does not owe its anti-microbial properties to bacteriolysis. The explanation offered by Wassermann and Citron, of the action of the watery extracts in hindering the anti-bacterial action of a serum, viz., that the suspended bacillary particles intervene in and prevent the bacteriolytic process, cannot, in this case, be regarded as altogether appropriate, unless the preparation of the bacilli for phagocytosis by the serum is regarded as a stage in an incomplete bacteriolysis. Neither can the immunity in the case mentioned be reasonably regarded as the result of the production of anti-aggressins, even if we assume that the natural and artificial aggressins are identical. Bail states that, at least in the case of typhoid and cholera, heating to 60° C., severely damages the aggressin, whereas in this case a temperature of 70° C. was employed. The conditions, therefore, for anti-aggressin production were not fulfilled in these experiments. It is, however, conceivable that an aggressin modified by heating might still be capable of stimulating to the formation of an anti-aggressin, just as a modified toxin or toxoid is capable of stimulating to antitoxin production.

The questions which are of great interest in relation to immunity generally, and especially to plague immunity, viz., whether the natural and artificial aggressins are the same or different bodies, their relation to toxins, and whether there exists an aggressin immunity *sui generis*, must be regarded as subjects still *sub judice*.

It has been shown in the preceding part of this paper that the protection conferred by anti-plague serum is little, if at all, dependent on antitoxic action. Nor does the humoral view of immunity which has been applied by many writers, among whom may be mentioned Kolle and Martini (1902), give a satisfactory explanation of its action since, as has been shown, no complete bacteriolysis *in vitro* can be demonstrated.

We must now, therefore, consider the part played by the serum in relation to phagocytosis, the importance of which as a factor in immunity is now universally admitted. Metchnikoff (1901), in reviewing the subject of the anti-infective or protective sera, says that the action may be direct on the micro-organism, and that it may be microbicidal, properly so-called, agglutinative, or fixative. In addition to these properties, and acting along with them, he upholds the view that these sera have a stimulating effect on the leucocytes.

The subject with especial relation to plague has been studied among others by Denys and Tartakowsky (1900), who attributed the anti-microbial action to the influence exerted by the serum on the process of phagocytosis.

Bordet and Gengou (1901) demonstrated the presence of substance sensibilisatrice in anti-plague serum by their method which is based on the observation that only sensitised bacilli are capable of removing alexin or complement from a fresh serum. The hæmolysis of sensitised red blood corpuscles is used as the test for free complement.

Employing this method, they showed that plague bacilli, which had been in contact with heated immune serum, removed the complement from fresh serum, and must, therefore, have been sensitised. These authors point out that certain micro-organisms, such as the B. pestis, are capable in this way of absorbing complement without undergoing any morphological changes, though the possibility of a physiological change having been effected is not excluded.

Markl (1903) made a very thorough investigation into the mechanism of the action of anti-plague serum. He found that all the phenomena could not be explained on the assumption that the serum had a bacteriolytic action, since bacteriolysis takes place only in the case of bacilli of low virulence. When he submitted plague bacilli *in vitro* to the action of an immune serum which had been reactivated by fresh normal serum, and made microscopical preparations at intervals of time, he found that the bacilli showed no marked changes such as would indicate that they had suffered any harm. On the other hand, if, in place of the normal serum, he used peritoneal lymph containing leucocytes, he found the leucocytes packed with bacilli which lost their normal staining reaction, and, at the end of 12 hours, in the case of bacilli of moderate virulence, the bacilli were represented only by dust-like detritus. Markl holds that, in the case where peritoneal lymph, rich in leucocytes, is used to reactivate the immune serum, not only is there great destruction of the bacilli within the leucocytes, but there occurs extra-cellular destruction of the bacilli which is inversely proportional to the virulence of the bacillus.

When a highly virulent plague culture is injected intraperitoneally the multiplication of the bacilli can be followed. There is very little exudate, in which only a few leucocytes are present, and there is a rapid increase in the number of bacilli followed by death in 24 hours.

When anti-plague serum has been injected into the animal 24 hours before the bacillus, there is observed, during the first hour, an increase in mononuclear leucocytes, around which the bacilli are clumped, some of them being englobed by the mononuclear leucocytes. After the first hour the polynuclear leucocytes multiply rapidly and englobe the bacilli, so that in three hours no free bacilli, and in six hours no bacilli either intra or extra-cellular, are observable. The exudate now consists entirely of polynuclear leucocytes. The animal in this case recovers.

When the number of bacilli is so great that the resistance conferred by the serum is insufficient to protect the animal, the bacilli which remain undevoured by the phagocytes continue to multiply till the death of the animal results.

The bacilli lying free and those within the mononuclear leucocytes retain their normal morphology and staining reactions, whereas, those within the polynuclear leucocytes tend to stain badly and to assume coccal-like forms.

In regard to the nature of the action of the serum in relation to phagocytosis, Markl says:—"To determine whether the injection of the immune serum has an influence on the cells of the body, or whether the bacilli are directly influenced, the following experiment was performed. A suspension of plague bacilli in normal salt solution was added to inactivated immune serum, and the mixture was placed three hours in the incubator. After centrifugalisation the clear fluid was pipetted from the sediment, which, after being several times washed with salt solution, was injected intraperitoneally into rats. These bacilli thus acted on by the immune serum behaved in the animal body exactly like bacilli which had been injected along with free immune serum. They were englobed by the phagocytes.

The phagocytosis occurred so rapidly and was so extensive that no doubt remains that the immune serum has a direct influence on the bacilli, entering into union with them and conferring on them a positive chemiotaxis." *

He concludes that in the case where bacilli of low virulence are injected extracellular bacteriolysis plays an important part, whereas,

* A discussion of the relation of Markl's work to the views of Douglas and Wright is avoided in this paper as of too controversial a nature.

where highly virulent bacilli are injected, phagocytosis is the only agency at work in their destruction.

The writer's experience and results are largely in agreement with Markl's, but, as already mentioned, in dealing with the subject of bacteriolysis *in vitro*, evidence of complete destruction of the bacilli, even of low virulence, could not be obtained. The results observed by Markl when the bacilli were injected into the peritoneum can also be obtained in the subcutaneous tissue. The writer has found that when a bacillus of high virulence is injected into the subcutaneous tissue of the abdomen, or where infection is caused by shaving the skin and rubbing the culture on the shaved area, *i.e.*, by the cutaneous method, an enormous multiplication of the bacilli takes place within the lymph spaces, which become distended with lymph in the vicinity of the point of inoculation. Microscopical preparations from this region show a complete, or almost complete, absence of leucocytes. On the other hand, if the animal has been immunised by an injection of anti-plague serum, given say 24 hours before the injection of the bacilli, instead of the diffuse indefinite swelling, or absence of any swelling, visible to the naked eye found in the unprepared rat, a well marked generally sharply circumscribed swelling is observed, and microscopical examination shows in it the presence of an enormous number of polymorphonuclear leucocytes, many of which are packed with bacilli. Animals in which this condition is observed either recover or die at a much later period than the controls.

Summary.

Toxins can be demonstrated in the filtrates of bouillon cultures of the B. pestis. These toxic substances are probably set free by the disintegration of the bacillus through autolysis, but, since Albrecht and Ghon and Markl were able to demonstrate their presence in young cultures, the possibility is not excluded that they are toxins in the narrower sense, *i.e.*, secretory products of the bacillus. The toxin is much more toxic for the rat and mouse than for the rabbit and guinea-pig. The toxicity for mice is destroyed by heating to 70° C. for 30 minutes.

Under certain conditions, the injection of toxin stimulates the animal body to the formation of antitoxin, and the experiments recorded here

appear to show that this antitoxin is capable of neutralising the toxin, either when mixed with it or when the serum is injected twenty-four hours before the toxin, and when the two substances are injected at different parts of the body.

It seems doubtful whether it will be possible to maintain a hard and fast line of demarcation between the toxins proper and the endotoxins. The injections of emulsions of bacilli from agar, as well as of the toxic filtrates from broth cultures, may, in certain cases, stimulate the animal organism to the production of an antitoxic serum as was shown in the Laboratory here, by Todd (1904), in the case of the B. dysenteriæ of Shiga. The view that it is a general biological law, as stated by Kolle, that the injection of endotoxins into the animal body does not stimulate to antitoxin production, cannot be unreservedly accepted, but must be subjected to further investigation.

Whether the toxin and antitoxin in the case of plague differs or not essentially from those obtained in the case of diphtheria and tetanus, it must be admitted that it is impossible to obtain them in anything like the same degree of concentration.

The neutralisation of toxin by the antitoxin *in vitro* is not due to the occurrence of a precipitin reaction. The serum of a horse giving a much greater precipitum with a particular toxin may possess a much lower antitoxic value than the serum of a horse which gives a much smaller precipitum with the same toxin. The fact that the neutralisation also takes place *in vivo* is against the view that a precipitin reaction plays a part in the phenomenon, for Rostoski, Michaelis and Oppenheimer (1902), showed that this reaction never takes place in the blood of the living animal.

So far as has been demonstrated experimentally, antitoxic and bacteriolytic actions play little part in the protection afforded by the anti-plague serum. The action of the serum can probably be best interpreted as follows:—The serum acts, not by any influence on the cells of the animal body, but directly on the bacilli, which, after being submitted to its action, no longer repel but rather attract the leucocytes, by which the microbes are then englobed and digested. This was first shown in the case of the B. pestis by Markl.

The change in the microbe has been expressed by stating that the immune serum converts the negative chemiotaxis of the bacillus into

a positive chemiotaxis. The substance producing the change has been variously designated as fixateur, substance sensibilisatrice, opsonin and incitor.

It is still an open question whether we are here dealing with a stage in an incomplete bacteriolysis in which the amboceptor acts as the intermediary between the bacillus and the leucocyte. The view is one which can be maintained with much reason. Certain organisms, such as the Cholera vibrio, when acted on by an immune serum containing amboceptor and complement, undergo bacteriolysis, which can be seen under the microscope, or estimated by the absence of growth. Other organisms, such as the B. pestis, are acted on by an immune serum, but in this case the solution or death of the bacillus does not take place outside the cells. That the organism has, however, suffered change is shown by the altered behaviour of the leucocytes to which we have just referred.

Whether the bacillus undergoes an extra-cellular bacteriolysis, or is digested after being phagocytosed, it must not be overlooked, that in both cases it is highly probable that the ultimate protection of the animal is effected by the phagocytes, which, in the former case, take up the toxic particles set free by bacteriolysis, in the latter, take up and digest the bacillus and retain the toxic particles liberated during the process.

When the extra-cellular or intra-cellular bacteriolysis is carried on with too great energy so that the leucocytes are not strong or numerous enough to deal with the toxic products, the death of the animal results.

The question as to whether anti-plague serum owes any of its protective power to the "stimulines" of Metchnikoff and the "anti-aggressins" of Bail must be decided by further investigations.

Conclusions.

1. That toxins can be obtained from broth cultures of the plague bacillus.

2. That these toxins are probably endotoxins, as the toxicity of the filtrate increases with the age of the culture, and becomes considerable only after many of the bacilli have been autolysed.

3. That the injection of large doses of these toxic filtrates is capable of stimulating the animal body to the formation of an antitoxin.

4. That the neutralisation of the toxin by antitoxin is not merely due to a precipitin reaction for (*a*) it occurs *in vivo* where no precipitin reaction takes place, and (*b*) a serum with little or no antitoxic properties may be capable of producing a marked precipitin reaction and *vice versa.*

5. That neither antitoxic nor bacteriolytic actions play much part in the protection afforded by an anti-plague serum.

6. That the anti-microbial action of an anti-plague serum is, as was first shown by Markl, almost, if not entirely, due to a direct action of the serum on the microbe, by means of which its negative chemiotaxis is converted into a positive chemiotaxis, so that it no longer repels but rather attracts the phagocyte. The substance in the serum producing this action is thermostable, and has been variously named fixateur, substance sensibilisatrice and incitor.

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A NOTE ON THE INFLUENCE OF THE CHEMICAL RAYS OF DAYLIGHT ON VACCINIA IN ANIMALS.

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In view of the observations made of late years on the action of red light in variola, it was thought that it might be of interest to ascertain, if possible, whether the red, or other rays of daylight, exerted any influence in cases of vaccinia; that is to say, with a disease the specific virus of which differs from that of variola probably only in that its pathogenic capabilities are modified, while vaccinia presents clinically many points of resemblance to variola.

As it was conceivable that any influence which rays of daylight might exert *in vivo* might also be exhibited on the virus outside the animal body, in the first instance experiments were made on vaccine material *in vitro*.

Experiments on vaccine emulsion in vitro.

Vaccine emulsions were subjected to the influence of variously coloured rays of daylight in the following manner:—

Vaccine pulp was collected in the usual way from a calf 120 hours after the animal had been vaccinated. A portion of this pulp was emulsified with four times its own weight of sterile distilled water, and was subjected to the chloroform process. A second portion of the pulp was emulsified with four times its own weight of a 50 % mixture of pure glycerine and sterile distilled water.

The chloroformed emulsion was partly used to fill seven small glass vessels of test tube shape each having a capacity of 5 c.c. Each tube was filled with vaccine and tightly corked.

One of each of these seven tubes was placed in a separate glass compartment. The first compartment was made of red glass, the second of yellow, the third of green, the fourth of blue, the fifth of violet, the sixth not coloured, and the seventh, also of uncoloured glass, was itself enclosed in a light-tight tin box.

The remainder of the chloroformed vaccine emulsion was put into *glass capillary tubes* and six of these filled capillary tubes were also placed in each of the seven above mentioned glass compartments. Capillary tubes were used in order that a thin column of vaccine emulsion, as well as the thicker columns of vaccine in the small test tubes, might be subjected to the various coloured rays.

The glycerinated emulsion was placed in small test tubes and glass capillary tubes in a similar manner and these were similarly placed in the compartments.

The compartments themselves were placed in a laboratory window facing north and were all equally subjected to the action of daylight throughout the day. Altogether vaccine from seven calves was treated in this manner, the exposure in the glass compartments lasting continually from the middle of July to the end of February. At the end of this time some rabbits and two calves were inoculated with all the seven vaccines of each compartment, a sample of each vaccine being taken from both a capillary tube and from one of the small test tubes.

It is needless to tabulate the result of these inoculations, for the vesiculations of both calves and rabbits failed to show any difference between the various portions of any one of the seven vaccines. Possibly the vaccine from the small test tubes gave rather better vaccination results than did the vaccine from the capillary tubes, but further than this no difference could be noted.

It would appear from these *in vitro* experiments that the resistance of the specific virus of vaccinia to the germicidal action of daylight is greater than the resistance usually shown by non-spore-bearing micro-organisms, and approximates more closely to the resistance of spore-bearing bacteria.

Experiments on Vaccinated Animals.

The animal experiments were conducted as follows:—Three rabbits, young animals—six to twelve weeks old—and as similar as possible in size and condition, were taken. Each animal had an area, roughly 4 × 5 millimetres, shaved on its back. The areas of each set of three

rabbits were inoculated in linear incisions with vaccine lymph from the same calf.

Immediately after inoculation the first rabbit was placed in a cage which had been closely covered with a double layer of red glazed cambric such as is used for photographic dark rooms. This covered cage was so situated that it received on its top and four sides direct daylight from sunrise to sunset. The red cover of the cage was only partially and momentarily removed twice in the 24 hours during the first 72 hours after the inoculation of the rabbit, for the purpose of placing the food inside the cage. After the first 72 hours the rabbit was removed for a few minutes to examine the condition of its vaccinated area, after which it was returned to the red light for a further 24 or 48 hours.

The second rabbit of the batch was, immediately after inoculation, placed in the dark, light being admitted to the cage momentarily only when feeding the animal. The rabbit was kept thus for 72 hours after inoculation, at the end of which time—as in the case of rabbit No. 1—it was removed for a few minutes to ascertain the condition of its vaccinated area. After examination it was replaced in the dark for 24 or 48 hours or more.

In experiments with red light in small-pox[1] any advantage which may have accrued in consequence of "red light treatment" may obviously have been due to the red light *qua* red light or to the fact that the chemical rays were occluded. By using a dark cage as an adjunct to the red light the advantage of the latter should be emphasised or detracted from as the case might be.

The third vaccinated rabbit was kept in such a way that it was in direct daylight during the daylight hours of the first 120 hours or more after its inoculation.

The cages of all three rabbits were arranged close together so that apart from the admission or occlusion of light rays, the animals were under the same conditions. The majority of the experiments were made at a time of year when it was daylight during more than 12 of the 24 hours. 31 sets of rabbits were thus treated (93 animals in all).

The results of these vaccinations were noted at varying intervals and are set forth in the accompanying Table.

The various degrees of development have been classified, somewhat

[1] *vide* Finsen, *Brit. Med. Journ.*, 1903, Vol. I. p. 1297, and *Lancet*, 1904, Vol. II. p. 1272. Schamberg, *Journ. Amer. Med. Assoc.*, 1903, Vol. XL. p. 1183, and 1904, Vol. XLIII. p. 1641. Ricketts and Byles, *Lancet*, 1904, Vol. II. p. 287, and 1904, Vol. II, p. 816.

roughly, into four classes. Class 1 includes all vesicles containing lymph of high virulence, Class 2 all vesicles containing lymph of secondary virulence, Class 3 vesicles containing lymph of virulence next below that of 2, and Class 4 all vesicles containing lymph of low virulence. Absence of vesiculation is recorded as 0. This classification takes no note of any sign other than the appearance of the vesicle itself.

The appearance of each areola was recorded and, according to its degree of intensity, was noted under one of three headings, the most extensive being 1, the medium 2, and that below the medium 3, while absence of areola was noted as 0.

TABLE.

	Class of areolae				Class of vesicles				
Nature of Exp.	1	2	3	0	1	2	3	4	0
Red light	8	8	8	7	23	1	2	4	1
Dark	11	14	4	2	17	1	5	8	0
Daylight	10	12	6	3	15	2	4	10	0

Dealing in the first instance with the tabulated results of the areolae, it will be observed that the largest number of the most severe areolae occurred in those animals protected from all light rays. Those animals which were exposed to red light had the smallest number of the severest type of areola, while the "daylights" had an intermediate number. Again, in the case of those animals which had least extensive areolae, if the "red lights" and the "daylights" are examined it will be seen that the former have the greatest numbers, whereas those animals protected from all light rays had a smaller number of mild areolae than had the "daylights." The same contradiction of results is seen if one examines the totals of those animals which showed no areolae, for of these the "red lights" are in the greatest number, and the "darks" in the least, the "daylights" being intermediate.

It is clear that in the case of these experiments no deduction can be drawn as to the action of any of the rays of daylight in the production of vaccination areolae, unless it be that the degree of areola is uninfluenced by the presence or absence of daylight.

Proceeding to the examination of the vesicles it will be seen that by taking the actual total numbers of the results, it would appear that a distinctly larger number of cases exposed to red light developed vesicles of a highly virulent type than did those cases exposed to daylight; while those cases left in the dark, and therefore also excluded from the chemical rays, gave a somewhat larger number of virulent vesicles than did the cases exposed to daylight.

These figures are corroborated by examining the numbers of vesicles of low virulence developed in red light, dark, and daylight respectively. It will be seen that the proportion of vesicles of low virulence was rather larger among the "daylight" than among the "dark" animals, and much larger than among the "red lights."

In order to gain additional information on this point three goats were vaccinated and treated in a manner similar to that adopted in the case of the rabbits. The goats were placed in red light, daylight, and dark respectively, and the results of the vaccinations were noted from the sixth to the tenth days. In respect to vesiculation the results were in complete accordance with the results of the experiments with rabbits, for the goat exposed to red light developed vesicles of first-rate quality, the goat left in the dark developed vesicles almost as good, while the goat left in daylight yielded only poor vesicles.

As a further experiment four more goats were vaccinated and treated in a manner similar to that of the former three, except that it was not found practicable to use red light. One of the animals was placed in the dark, and the other three were tethered during the course of the experiment in an unshaded field. This experiment was carried out in the middle of June, 1906.

On the sixth, eighth, and tenth days after vaccination the goat excluded from daylight showed exceptionally good vesicular development, while none of the three animals kept in daylight showed any appearance of real vesiculation, dried lines of crust only appearing along the lines of inoculation.

A few days later a further experiment was made with two calves. Each of these calves was vaccinated from the same batch of vaccine lymph, following the procedure of the former experiments. One of the calves was placed in the dark, while the other was kept in the open field.

On the fifth day after inoculation the calf protected from daylight showed well-developed, typical vesicles, while the calf kept in the field failed to show more than the merest trace of vesiculation. This second animal was kept under observation under the same conditions for three weeks, during which time no further vesiculation appeared.

From these experiments it would seem at least to be strongly indicated:—

(1) That chloroform water emulsions, and glycerine water emulsions of vaccine lymph *in vitro* are not appreciably affected with regard to their potency by exposure to or protection from daylight. The vaccine

virus indeed would appear to resemble in this respect ordinary bacterial spores rather than the usual non-spore-bearing bacteria.

(2) The development of the areola in vaccinated rabbits is apparently unaffected by the exposure of these animals to the chemical rays of daylight, or by protecting them from such rays.

(3) That vaccinia in rabbits, goats, and probably in calves, *as a specific disease* is influenced in such a way by the prolonged exposure of the vaccinated animals to the chemical rays of daylight that its development is prevented to a greater or lesser extent.

Should this last point be established, the advantage in a vaccine establishment of protecting the animals used for the production of vaccine lymph from the rays of daylight is obvious.

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On Voges and Proskauer's Reaction for Certain Bacteria.

By ARTHUR HARDEN, D.Sc., Ph.D.

(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—Read February 1, 1906.)

(From the Chemical Laboratory Lister Institute.)

In 1898 Voges and Proskauer* described a new colour reaction which they had observed in the case of a bacillus, isolated by Voges and grown in a medium containing sugar. When potash was added and the tube allowed to stand for 24 hours or longer at room temperature, a beautiful fluorescent colour, somewhat similar to that of a dilute alcoholic solution of eosin, formed in the culture fluid, particularly at the open end of the tube exposed to the air. The reaction was found to be specific to the bacillus in question, and was not given by any of the other organisms isolated in the course of the investigation upon which they were engaged, nor by the *B. coli communis*, so that it afforded a most valuable means of differentiation for the inhabitants of the intestine. Durham† and How‡ have also employed this reaction for the discrimination of intestinal bacteria, and MacConkey,§ in confirmation of Durham, has found that out of a large number of bacteria which were tested only three gave the reaction, these being *B. lactis aerogenes* (Escherich), *B. capsulatus* (Pfeiffer), and *B. cloacæ* (Jordan).

The examination of the products formed by *B. lactis aerogenes* from glucose‖ has shown that acetylmethylcarbinol, $CH_3.CO.CH(OH).CH_3$, and 2·3-butyleneglycol, $CH_3.CH(OH).CH(OH).CH_3$, are both present in the medium in which this has been cultivated in the presence of glucose. The acetylmethylcarbinol has not as yet been isolated in the pure state, but is present in the aqueous distillate obtained by distilling the culture medium. This distillate and the glycol were, therefore, treated with caustic potash in order to ascertain whether either of them was the cause of the reaction just described. Neither of these substances produces the characteristic fluorescent coloration with potash alone, but when peptone water is also added, acetylmethylcarbinol gives the reaction after standing for about 24 hours, whilst the glycol does not react in this way even on standing. The coloration was produced in the characteristic manner described by

* 'Zeitschr. f. Hyg.,' 1898, vol. 28, p. 20.

† 'Journ. of Experimental Medicine,' 1900—1901, p. 354.

‡ 'Centralbl. f. Bakter.,' 1904, vol. 36, p. 484.

§ 'Journ. of Hyg.,' 1905, vol. 5, 349.

‖ Harden and Walpole.

Voges and Proskauer, commencing at the open end of the tube exposed to the air. This suggests oxidation as a factor in the phenomenon, and as acetylmethylcarbinol is very readily converted by oxidation into diacetyl, $CH_3.CO.CO.CH_3$, this substance was tested. Diacetyl yields the fluorescent red coloration with peptone water and caustic potash in a few minutes, and by its aid a much greater depth of colour can be obtained than that observed with bacterial cultures.

Voges and Proskauer's reaction, therefore, appears to be due to acetylmethylcarbinol, which is formed by the action of the bacteria on the glucose of the medium. In the presence of potash and air this is oxidised to diacetyl, which then reacts with some constituent of the peptone water. That diacetyl is the active substance and not *p*-xyloquinone, $C_6H_2O_2(CH_3)_2$, which is readily formed from it by the action of alkalis, is shown by the fact that if the diacetyl be allowed to stand for some time with potash solution, and peptone water be then added, no reaction occurs.

B. cloacæ (Jordan), which gives Voges and Proskauer's reaction, was also found to yield acetylmethylcarbinol, which was recognised by its power of reducing Fehling's solution in the cold and of yielding the characteristic phenylosazone of diacetyl with phenylhydrazine.

Acetylmethylcarbinol has also been observed as a product of the action of certain other bacteria on glucose. Thus, Grimbert* found that it is produced by *B. tartricus*, and Desmots† that it is also formed by the various bacilli of the mesentericus group and by *B. subtilis* and *Tyrothrix tenuis*. These bacteria should, therefore, give Voges and Proskauer's reaction, and, as a matter of fact, *B. mesentericus fuscus*, the only one which has so far been examined, gives the reaction quite characteristically when grown in peptone water containing 2 per cent. of glucose.

A number of other bacteria are being examined and attempts are also being made to ascertain what constituent of the peptone water it is that reacts with the diacetyl.

* 'Compt. Rend.,' 1901, vol. 132, p. 706.
† 'Compt. Rend.,' 1904, vol. 138, p. 581.

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The Alcoholic Ferment of Yeast-Juice.

By ARTHUR HARDEN, D.Sc., Ph.D., and WILLIAM JOHN YOUNG, M.Sc.

(Communicated by Dr. C. J. Martin, F.R.S. Received December 8, 1905,—Read February 1, 1906.)

(From the Chemical Laboratory, Lister Institute.)

1. *Effect of the addition of Boiled and Filtered Yeast-juice on the Fermentation of Glucose Produced by Yeast-juice.*

In the course of some experiments on the action of various proteids on the fermentative activity of yeast-juice, it was observed that the alcoholic fermentation of glucose by yeast-juice is greatly increased by the addition of yeast-juice which has been boiled and filtered, either when fresh or after having undergone autolysis, although this boiled liquid is itself incapable of setting up fermentation. Thus, the total fermentation produced by yeast-juice acting on excess of glucose is, as a rule, doubled by the addition of an equal volume of the boiled juice, and a further increase is produced when a greater volume is added, the sugar concentration being kept constant.*

A similar observation was previously made by Buchner and Rapp† in a single experiment (No. 265).

The following table embodies a few of the results obtained, the yeast-juice being prepared and the amount of carbon dioxide evolved being estimated by

* Harden and Young, Preliminary Note, 'Proc. Physiol. Soc.,' 1904, vol. 32, November 12.

† 'Ber.,' 1899, vol. 32, p. 2093.

the method previously employed by the authors.* In every case the concentration of sugar was kept constant, and both in these and all the fermentation experiments described in this paper, toluene was added as an antiseptic.

Table I.—Effect of the Addition of Boiled Yeast-juice on the Total Fermentation of Glucose by Yeast-juice.

No.	Juice.	Water.	Boiled juice.	Glucose.	Time.	Carbon dioxide.
	c.c.	c.c.	c.c.	grammes.	hours.	gramme.
1	25	25	0	5	72	0·137
	25	0	25	5	72	0·378
2	20	20	0	4	44	0·115
	20	0	20	4	44	0·363
3	25	0	0	2·5	40	0·370
	25	0	25	5	40	0·620
4	20	40	0	6	42	0·458
	20	0	40	6	42	0·858
5	25	25	0	5	44	0·346
	25	0	25	5	44	0·709
6	25	25	0	5	48	0·110
	25	0	25	5	48	0·216
7	25	25	0	5	60	0·273
	25	0	25	5	60	0·466
8	25	25	0	5	120	0·424
	25	0	25	5	120	0·959
9	25	25	0	5	72	0·414
	25	20	5	5	72	0·546
	25	10	15	5	72	0·735
	25	5	20	5	72	0·810
	25	0	25	5	72	0·924
10	25	25	0	5	70	0·246
	25	0	25	5	70	0·356
	25	50	0	7·5	70	0·180
	25	0	50	7·5	70	0·431
	25	75	0	10	70	0·141
	25	0	75	10	70	0·515

In Experiments 1 to 5 the juice added had been autolysed before being boiled; in Nos. 6 to 8 the added juice was boiled as soon as it had been prepared. Experiments 9 and 10 show that each successive addition of boiled juice, from 0·2 to 3 volumes, produces a further increase in the amount of the fermentation.

A similar effect is produced, (1) By the precipitate produced in boiled yeast-juice by the addition of 3 volumes of alcohol (Experiment 1, Table II); (2) By the liquid formed by the autoplasmolysis of yeast, when it is allowed to stand at the air temperature for some time (Experiments 2 and 3, Table II); (3) By the liquid obtained by boiling Buchner's "Aceton-Dauerhefe" with

* Harden and Young, 'Ber.,' 1904, vol. 37, p. 1052.

water (Experiment 4, Table II). Further, yeast killed by acetone and ether (Aceton-Dauerhefe) reacts with boiled juice in the same way as does yeast-juice (Experiment 5, Table II).

Table II.—Effect of Various Substances in Increasing Alcoholic Fermentation.

No.	Yeast-juice.	Addition.	Glucose.	Time.	Carbon dioxide.
	c.c.			hours.	gramme.
1	25	25 c.c. water	5	48	0·110
	25	25 c.c. water + precipitate by 75 per cent. alcohol from 25 c.c. boiled fresh juice	5	48	0·268
	25	Filtrate from 25 c.c. boiled fresh juice + 3 volumes alcohol, made to 25 c.c.	5	48	0·141
	25	25 c.c. water + precipitate from 25 c.c. boiled old juice by 75 per cent. alcohol	5	48	0·286
2	25	25 c.c. water	5	72	0·070
	25	25 c.c. autoplasmolysed yeast-juice, made neutral	5	72	0·189
3	25	25 c.c. water	5	72	0·084
	25	25 c.c. autoplasmolysed yeast-juice, made neutral	5	72	0·172
	25	25 c.c. water	5	72	0·475
4	25	25 c.c. aqueous infusion of 2 grammes Aceton-Dauerhefe	5	72	0·625
5	2 grammes Aceton-Dauerhefe	40 c.c. water	4	48	0·062
	,,	20 c.c. water + 20 c.c. boiled juice	4	48	0·136

2. *Dialysis of the Boiled Juice.*

The constituent of the boiled and filtered juice to which this effect is due is removed when the liquid is dialysed in a parchment tube, leaving an inactive residue. In the experiments detailed in the following table (Table III, Experiments 1, 2 and 3) the effect of the addition of boiled juice is compared with that produced by the residue and dialysate respectively.

In Experiment 4, the unboiled juice was dialysed, and the fact that the dialysate had a similar effect to a boiled juice shows that the active constituent exists in the original yeast-juice and is not formed during the boiling.

Table III.—Dialysis of Boiled Yeast-juice. 25 c.c. yeast-juice + 5 grammes glucose + toluene.

No.	Water.	Boiled juice.	Residue.	Dialysate.	Time.	Carbon dioxide.
	c.c.	c.c.	c.c.	c.c.	hours.	gramme.
1	25	0	0	0	48	0·253
	0	25	0	0	48	0·561
	0	0	25	0	48	0·264
2	25	0	0	0	48	0·268
	0	25	0	0	48	0·497
	0	0	25	0	48	0·276
3	25	0	0	0	72	0·113
	0	25	0	0	72	0·334
	0	0	25	0	72	0·189
	0	0	0	25	72	0·334
4	25	0	0	0	48	0·154
	0	0	0	25	48	0·251

3. *Dialysis of Yeast-juice.*

The facts above detailed suggested the possibility of dividing yeast-juice into two fractions by dialysis; an inactive residue and a dialysate which, although itself inert, would be capable of rendering this residue active.

This was experimentally realised by filtering the juice through a Martin gelatin filter.*

This method of rapid dialysis was chosen because the yeast-juices at our disposal lost their activity too rapidly to permit of the ordinary process of dialysis through parchment being carried out. Either a 10- or a 7·5-per-cent. solution of gelatin was used to impregnate the Chamberland filter and the filtration was carried out under a pressure of 50 atmospheres.

Only a portion of the juice placed in the filter was actually filtered, the remainder being simply poured out of the case as soon as a sufficient quantity of filtrate had passed through. The residue adhering to the candle, which consisted of a brown viscid mass, was dissolved in water and made up to the volume of the juice filtered. Glucose was then added and one portion incubated at 25° with an equal volume of sugar solution and a second portion with an equal volume of the filtrate or of a boiled juice, containing an equal amount of glucose. Before incubation the carbon dioxide was pumped out of all the solutions. The filtrate was invariably found to be quite devoid of fermenting power, none of the enzyme having passed through the gelatin.

* 'Journ. Physiol.,' 1896, vol. 20, p. 364.

The results (Table IV) show that in this way an almost inactive residue can be obtained which is rendered active by the addition of the filtrate (Experiments 1, 2, 3) or a boiled juice (Experiment 4).

Table IV.—Filtration of Yeast-juice through the Martin Gelatin Filter. 15 c.c. residue + 3 grammes glucose + toluene.

No.	Water.	Filtrate.	Boiled juice.	Time.	Carbon dioxide.
	c.c.	c.c.	c.c.	hours.	gramme.
1	15	0	0	48	0·000
	0	15	0	48	0·035
2	15	0	0	60	0·001
	0	15	0	60	0·051
3	15	0	0	60	0·008
	0	15	0	60	0·064
4	15	0	0	60	0·024
	0	0	15	60	0·282

The total fermentations observed even in the presence of the filtrate are very low, this being, at all events in part, due to the fact that in this series of experiments the original juices themselves happened to be of low fermenting power.

In a second set of experiments (Table V) a smaller quantity of juice was placed in the filter and the filtration was continued until no more liquid would pass through. The residue was then washed several times by adding water and forcing it through the filter. The time occupied in this process varied greatly with different juices, the limits for the filtration and washing of 50 c.c. of juice, using two filters simultaneously, were about 6 to 12 hours. The carbon dioxide was not estimated by absorption in potash as in the previous cases, but was collected and measured over mercury, by means of the apparatus described later on, the object of this procedure being to ascertain not only the total amount of carbon dioxide produced, but the rate and duration of the evolution. The residue was dissolved in water and made to the same volume as the original juice, and the filtrate was evaporated down to the same volume. All the solutions were saturated with carbon dioxide at the temperature of the bath (25°) before the measurements were commenced, and the observations were continued until all fermentation had ceased.

The boiled juice added in Experiments 1, 3 and 4 (Table V) was obtained. by boiling a portion of the same preparation as was used for the filtration. The carbon dioxide is expressed in cubic centimetres under atmospheric conditions.

Table V.—Filtration of Yeast-juice through the Martin Gelatin Filter.

No.	Vol. of juice filtered.	Wash water.	Residue.	Filtrate.	Boiled juice.	Glucose.	Carbon dioxide
	c.c.	c.c.	c.c.	c.c.	c.c.	grammes.	c.c.
1	75	200	25	0	0	2·5	10·4
			25	0	25	5	396·3
2	80	260	20	0	0	2	8·3
			20	20	0	4	90·2
3	100	250	25	0	0	2·5	0·4
			25	0	25	5	268
4	50	200	25	0	0	2·5	0·9
			25	0	25	5	192

The process of filtration does not always produce an inactive residue, as on several occasions the residue after very thorough washing has been found to retain a considerable amount of activity. No reason has yet been found for this and it has not yet been ascertained whether it is due to some peculiarity in the particular specimen of juice or in the special filter employed.

It is of interest to note that in Experiment 2 (Table V) the residue alone gave 8·3 c.c. of carbon dioxide in 3 hours, the amount evolved in the last hour being only 0·1 c.c. At the close of this period the liquid still contained the alcoholic enzyme, since on the addition of 20 c.c. of the filtrate, fermentation recommenced and continued for many hours.

These two sets of experiments (Tables IV and V) show that the fermentation of glucose by yeast-juice is dependent upon the presence of a dialysable substance which is not destroyed by heat.

4. *Analysis of the Effect of the Addition of Boiled Juice upon the Fermentation of Glucose by Yeast-juice.*

In order to compare the course of the fermentation in the presence and in the absence of boiled yeast-juice, experiments were carried out in which the rate of evolution of carbon dioxide was observed in each case throughout the whole period of activity of the juice, which, as a rule, in presence of an excess of sugar, lasts for about 48 to 60 hours.

For this purpose the fermentation was allowed to proceed in a 100 c.c. flask, kept at the constant temperature of 25° by immersion in a thermostat, and connected with an azotometer, in which the gas was collected over mercury. The gas in the fermentation flask was maintained at a constant pressure, as nearly as possible that of the atmosphere, by keeping the mercury

in the reservoir at a fixed level, by means of a syphon dipping into a small beaker. The volume of the gas was read on the azotometer without disturbing the mercury reservoir and was reduced to atmospheric pressure by means of a calibration curve. Since yeast-juice readily becomes supersaturated with carbon dioxide, the contents of the flask were vigorously shaken before each reading of the volume of gas. Before the observations were commenced the liquids were brought to the temperature of the thermostat, and were saturated with carbon dioxide. In all comparative experiments the concentration of glucose was the same.

When the rates of evolution of carbon dioxide from (1) a solution of glucose in yeast-juice, and (2) a similar solution to which boiled and filtered yeast-juice has been added are compared, it is found that two phenomena are concerned in the production of the increased fermentation in the presence of boiled yeast-juice.

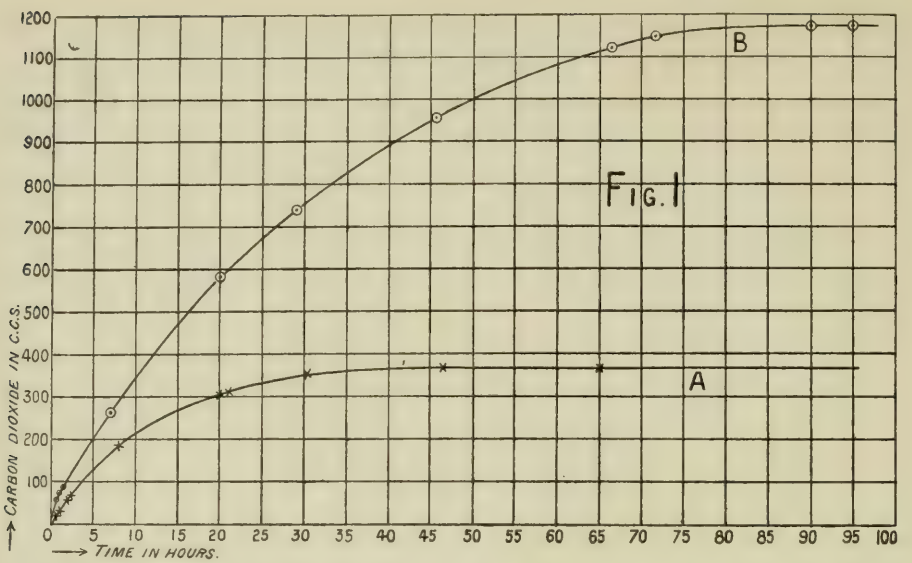
(*a*) An initial rapid evolution of carbon dioxide is produced, which soon diminishes until a rate is attained which remains nearly constant for several hours and is usually, but not invariably, approximately equal to that given by an equal volume of the same yeast-juice and glucose to which no addition has been made.

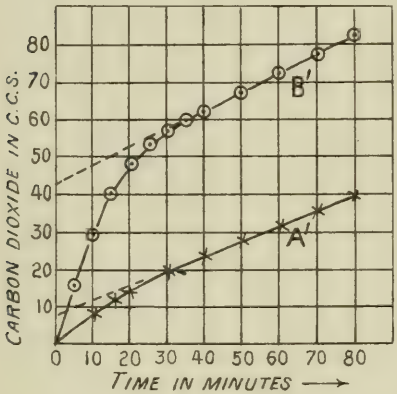
(*b*) The fermentation rate diminishes more slowly, so that the fermentation continues for a longer period. The greater proportion of the total increase is usually due to this second phenomenon.

The results obtained in a typical experiment of this kind are shown in Fig. 1. The initial period of the evolution is plotted separately (Curves A′ and B′) on a larger scale.

Curves A and A′ in which the evolution of carbon dioxide is plotted against time represent the course of a fermentation with 25 c.c. yeast-juice + 25 c.c. water + 5 grammes glucose + toluene. The rate to begin with is 48 c.c. per hour, but rapidly decreases until it becomes equal to 24 c.c. per hour, at which it remains almost constant for about 5 hours, gradually decreasing until, after the expiration of about 40 to 45 hours, fermentation ceases. The total evolution amounted to 369 c.c. under atmospheric conditions.

Curves B and B′ refer to 25 c.c. of the same yeast-juice + 25 c.c. of a boiled yeast-juice + 5 grammes glucose + toluene. The initial rate is much higher, 168 c.c. per hour, but this falls gradually in the course of 40 minutes to 30 c.c. per hour. This rate of 30 c.c. per hour falls off much less rapidly than that in Experiment A, the fermentation continuing for about 80 to 85 hours and yielding in all 1174 c.c. of carbon dioxide. It is important to bear in mind that these curves represent the gradual disappearance of the fermenting power





of the liquid, and not the diminution of the amount of fermentation with diminishing concentration of sugar, an excess of this substance being present throughout.

A comparison of the two curves shows very clearly the two factors involved in the great increase in Experiment B: (1) The initial rapid evolution, and (2) the prolongation of the fermentation.

5. *The Initial Period of Rapid Evolution of Carbon Dioxide.*

This is a very striking phenomenon, and a typical example is illustrated in fig. 2 in which the curves show the course of the evolution of carbon dioxide (total volume evolved plotted against time) during two hours in the case of:

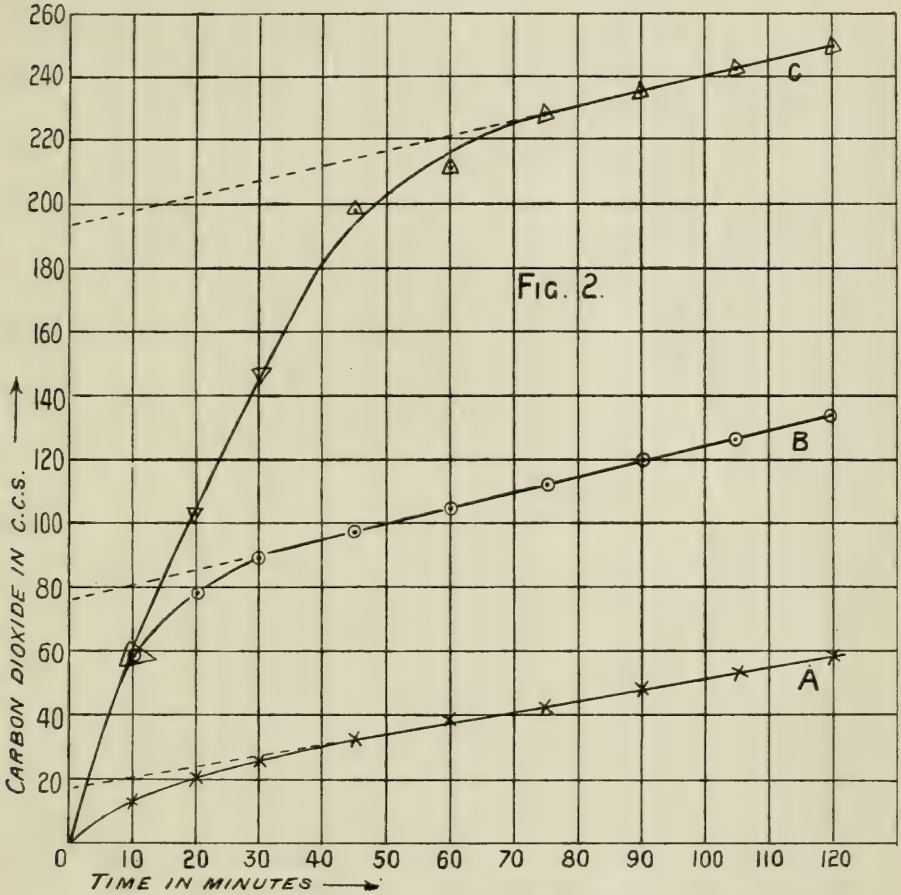
A. 25 c.c. yeast-juice + 75 c.c. water + 10 grammes glucose + toluene.

B. 25 c.c. yeast-juice + 50 c.c. water + 25 c.c. boiled autolysed yeast-juice + 10 grammes glucose + toluene.

C. 25 c.c. yeast-juice + 75 c.c. boiled autolysed yeast-juice + 10 grammes glucose + toluene.

In B and C the initial rates are almost equal (58 c.c. in 10 minutes) and much greater than in A (14 c.c. in 10 minutes). In B the rate rapidly falls

off whilst in C it diminishes much more slowly. A similar initial period is also observable in A, but is not nearly so marked.



The extra quantity of carbon dioxide evolved in this initial period may be calculated by subtracting the amount corresponding with the constant rate which is finally attained from the total amount observed. This is done graphically in fig. 2 by continuing the straight line representing the constant rate back to the axis of ordinates. The following numbers are thus obtained: for A, 16·6; for B, 75·4; for C, 192·9.

The amounts due to the addition of boiled juice are therefore: for 1 volume in B, $75{\cdot}4 - 16{\cdot}6 = 58{\cdot}8$; for 3 volumes in C, $192{\cdot}9 - 16{\cdot}6 = 176{\cdot}3 = 3 \times 58{\cdot}8$.

The extra amount of carbon dioxide is, therefore, directly proportional to the volume of boiled juice added.

6. *Production of the Initial Rapid Evolution of Carbon Dioxide by the Addition of Phosphates.*

As the result of a large number of attempts to isolate the constituent of boiled juice which brings about the increase in fermentation, it was found that whenever an increase was produced phosphoric acid in the form of a soluble phosphate was present. The effect of the addition of soluble phosphates to yeast-juice was, therefore, examined and it was found that a well-marked initial rapid evolution of carbon dioxide was thus produced. Since, moreover, the boiled juices employed invariably contained phosphates, precipitable by magnesia mixture, there can be no doubt that it is to the presence of these that this initial phenomenon is due. Quantitative estimations revealed the somewhat surprising fact that the extra quantity of carbon dioxide evolved in the initial period when a phosphate or a boiled juice is added, corresponds with the evolution of one molecular proportion of carbon dioxide for each atom of phosphorus added in the form of phosphate.

In order to obtain accurate results with solutions of sodium or potassium phosphate, the fact that these absorb carbon dioxide must be taken into consideration. Solutions of the dihydrogen salts of potassium and sodium are too acid to be employed and the monohydrogen salts or a mixture of these with the dihydrogen salts were always used. In every case the liquid before being added to the yeast-juice was saturated with carbon dioxide at the temperature of the bath, and the volume of carbon dioxide liberated by the addition of excess of hydrochloric acid was ascertained in an aliquot portion.

At the close of the fermentation the fermented liquid was acidified and the residual combined carbon dioxide measured, the difference between this and the original amount being subtracted from the amount evolved during the fermentation.

The results are more precise when the yeast-juice employed is an active one, since when the fermenting power of the juice is low the initial period becomes unduly prolonged and the calculation of the extra amount of carbon dioxide is rendered uncertain. The equivalence of the carbon dioxide and phosphate is established by the results contained in the following Table VI. Column 1 gives the observed amount of extra carbon dioxide calculated as described above and reduced to grammes, and Column 2 the equivalent of the phosphate added, this being estimated by precipitation with magnesium citrate mixture in the boiled juice or phosphate solution.

In Experiments 1 to 7 boiled juice was added; in 8 to 14 a solution of sodium or potassium phosphate.

The maximum rate attained during the initial period is from five to eight

times as high as the constant rate attained after the evolution of the carbon dioxide equivalent to the phosphate present.

Table VI.—Equivalence of Extra Carbon Dioxide Evolved during the Initial Period, and Phosphate added.

Experiments.	Grammes of carbon dioxide.		Experiments.	Grammes of carbon dioxide.	
	Column I—Observed.	Column II—Calculated from phosphate.		Column I—Observed.	Column II—Calculated from phosphate.
1	0·090	0·086	8	0·196	0·197
2	0·054	0·055	9	0·066	0·065
3	0·058	0·051	10	0·057	0·061
4	0·060	0·049	11	0·056	0·061
5	0·106	0·112	12	0·059	0·061
6	0·103	0·101	13	0·068	0·070
7	0·113	0·112	14	0·071	0·070

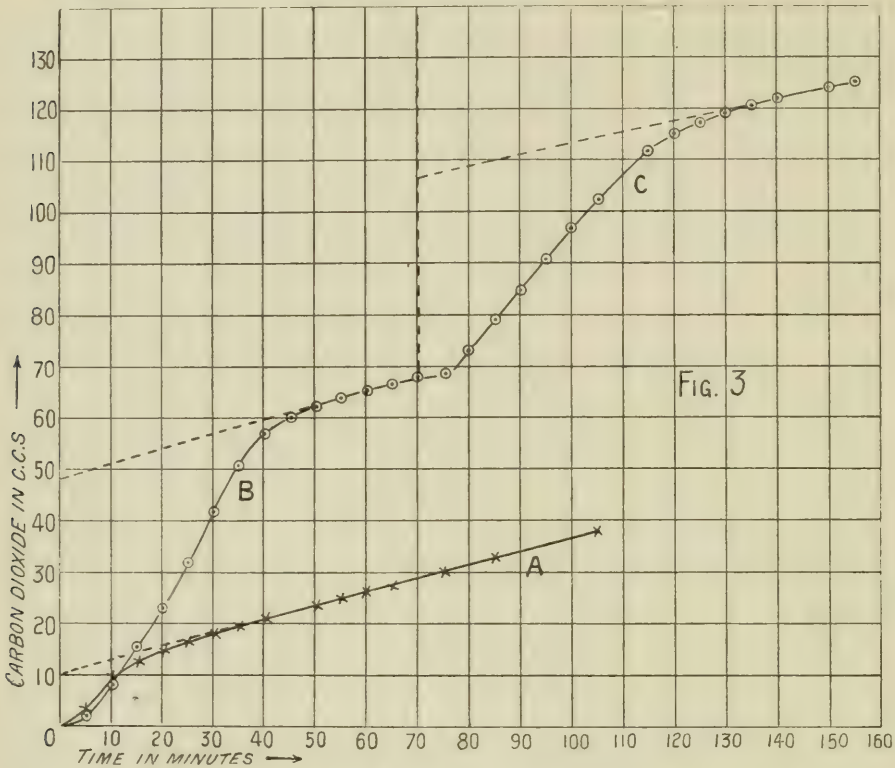
At the commencement of the period when sodium or potassium phosphate solution has been added, the rate only gradually acquires its maximum value and sometimes it only attains this maximum after a considerable interval.

This phenomenon is occasionally observed in the fermentation produced by yeast-juice without the addition of phosphate, and also sometimes occurs, but to a much smaller extent, when boiled juice is added. It is well shown in Curve B, fig. 3, which represents the fermentation produced by 25 c.c. yeast-juice + 25 c.c. of a 0·06 molar solution of sodium phosphate + 5 grammes glucose + toluene. The cause of this period of induction has not yet been ascertained.

7. *Limit of the Action of Phosphate.*

If the fermentation in presence of phosphate be allowed to continue until the steady rate is attained and a second quantity of phosphate be then added, a second period of rapid evolution of carbon dioxide sets in and proceeds in a similar manner to the first. This is shown in Curves B and C, fig. 3, which represent the effect of the successive addition of two quantities of 5 c.c. of 0·3 molar sodium phosphate to 25 c.c. yeast-juice + 20 c.c. water, in presence of 10 per cent. glucose. Curve A represents the fermentation in absence of added phosphate. The phosphate solution employed was a mixture of five molecules of NaH_2PO_4 with one molecule of Na_2HPO_4 and no correction for combined carbon dioxide was required. The extra amount of carbon dioxide evolved after each addition is the same, and is equivalent, as

already stated, to the phosphate added. The equality is shown graphically in the curve and the equivalence in Experiments 13 and 14, Table VI.



This process cannot, however, be repeated indefinitely, as after a certain limit is reached the reaction no longer occurs and with a large excess the fermentation is stopped. The exact limit appears to vary both with the nature of the phosphate added and with the particular specimen of yeast-juice employed. The greatest amount of carbon dioxide hitherto obtained in this way from 25 c.c. of yeast-juice is about 0·45 gramme (230 c.c.), which was observed on two occasions, once after the addition of four volumes of boiled juice, and again after the addition of 50 c.c. of a solution of a mixed magnesium potassium phosphate yielding with magnesia mixture 1·187 grammes of magnesium pyrophosphate.

When a specimen of yeast-juice has been incubated until it will no longer ferment sugar, it is not affected by the addition of phosphate.

The fact that the extra carbon dioxide calculated in this way is equivalent to the phosphate present, suggests the superposition of two actions. Whether this is to be explained by the presence of two distinct enzymes or simply by the increased activity of a single enzyme remains to be decided.

8. *Products of Fermentation in the Presence of Phosphate.*

The carbon dioxide evolved during the initial period after the addition of a phosphate is the product of a true alcoholic fermentation of the glucose, in which alcohol and carbon dioxide are produced in equivalent amounts. This was proved in the following way. Twenty-five cubic centimetres of a solution containing 2·5 grammes of glucose and 5 c.c. of a 0·3 molar solution of potassium phosphate were added to 25 c.c. of yeast-juice; the mixture was incubated and the carbon dioxide collected and measured.

As soon as the rate of evolution had become constant, a further addition of 10 c.c. of 0·3 molar phosphate solution was made and the fermentation again continued until the rate had become constant. The gas evolved was tested and found to be carbon dioxide. The total amount evolved during the experiment, which lasted for 2 hours 10 minutes, was 163·4 c.c. at 19°·6 and 758·6 mm. or 0·291 gramme, the equivalent of the phosphate added being 0·196 gramme. The liquid was then distilled with steam and the alcohol estimated in the distillate, 1·312 grammes being found to be present. Twenty-five cubic centimetres of the original juice were found to contain 0·983 gramme of alcohol and therefore $1·312 - 0·983 = 0·329$ gramme were formed by the fermentation of the sugar. The ratio of alcohol to carbon dioxide produced is therefore $0·329/0·291 = 1·13$, which agrees well with the ratio previously found by similar methods for the fermentation of glucose by yeast-juice.* The theoretical ratio is 1·04.

Lactic acid and acetic acid were also estimated in the original juice and after fermentation in presence of phosphate, but only a very small variation was observed. Twenty-five cubic centimetres of juice gave before fermentation 0·122 gramme of zinc lactate and 0·083 gramme of acetic acid, and after fermentation 0·102 gramme of zinc lactate and 0·072 gramme of acetic acid.

9. *Fate of the Phosphoric Acid.*

When the fermented liquid is boiled and filtered almost the whole of the phosphorus present is found in the filtrate, but it is nearly all in a form which is not precipitated by ammoniacal magnesium citrate mixture.

In the following experiment three quantities of 25 c.c. of yeast-juice were taken:—

A. Hot water was added, the solution heated in a boiling water-bath and the coagulate filtered off and well washed.

B. Ten cubic centimetres of a 30 per cent. glucose solution and 10 c.c. of

* Harden and Young, 'Ber.,' 1904, vol. 37, p. 1052.

0·3 molar potassium phosphate solution were added and the liquid at once heated to the boiling point, filtered, and the coagulate washed.

C. The same additions were made as to B and the liquid then fermented until the close of the initial period, after which it was heated and filtered like the others.

The total phosphorus was then estimated in each of the coagulates and in each of the filtrates, and the phosphorus precipitated by magnesium citrate in each of the three filtrates. The estimations of total phosphorus were made by heating with sulphuric and nitric acids until colourless, diluting and precipitating with magnesium citrate mixture in presence of excess of ammonia.

The following were the results obtained, the numbers representing the grammes of magnesium pyrophosphate per 25 c.c. of juice.

Table VII.

	A. Original juice.	B. Juice + phosphate. Not fermented.	C. Juice + phosphate. Fermented.
Coagulate	0·053	0·057	0·072
Filtrate—			
(*a*) Precipitated by Mg citrate	0·126	0·480	0·070
(*b*) Not precipitated by Mg citrate ...	0·271	0·282	0·679
Total	0·450	0·819	0·821

The amount of phosphate added was equivalent to 0·372 gramme of magnesium pyrophosphate.

A number of other results are given to show the extent to which phosphate is converted into the non-precipitable form by this reaction. All the estimations were made by boiling and filtering the fermented liquid immediately upon the close of the initial period. As before the numbers represent grammes of magnesium pyrophosphate obtained from 25 c.c. of juice.

The form in which this non-precipitable phosphorus is actually present in the fermented liquid, and in the liquid which has been boiled and filtered, has not yet been ascertained with certainty. Experiments which are still in progress, however, appear to indicate that it exists in combination with glucose, probably in the form of a phosphoric ester.

Table VIII.—Conversion of Phosphate into the Non-precipitable Form by Yeast-juice and Glucose.

	Phosphate added.	Precipitable phosphate in filtrate.	Non-precipitable phosphate in filtrate.
1	0·553	0·066	1·032
2	0·490	0·090	0·832
3	0·250	0·054	0·685
4	0·488	0·091	1·040
5	0·495	0·088	0·881

The question as to whether the entire phenomenon of the fermentation of glucose by yeast-juice depends on the presence of phosphates has not yet been definitely decided. The addition of phosphate undoubtedly produces a larger increase in the total fermentation than is simply due to the equivalent amount of carbon dioxide evolved in the initial period. The extent of this increase appears to vary very considerably with different specimens of yeast-juice, but the prolongation of the fermentation is not so great as is caused by boiled fresh juice. This question can only be satisfactorily settled by ascertaining whether the addition of a phosphate to the perfectly inactive residue obtained from a juice by filtration through a gelatin filter is sufficient to restore its fermenting power in the same way as the filtrate or a boiled juice. Experiments on this point are in progress, but no decisive result has as yet been obtained, and all discussion of this point will best be deferred until these are completed.

Various other points of interest raised in the course of the investigation, and the study of the relation of these phenomena to the fermentation of glucose by living yeast, are also occupying our attention.

A short outline of the main conclusions arrived at in the foregoing paper, has been previously published in the form of two preliminary communications, without any experimental details.* After the appearance of these notes, Buchner and Antoni† repeated and confirmed a number of the experiments dealing with the effect of boiled juice and of phosphates on the total fermentation, and with the separation of the juice by dialysis into an inactive residue and a dialysate capable of rendering it active. Buchner and Antoni were able, with the more stable juice at their disposal, to carry out the dialysis in the ordinary way for 24 hours and in this manner to confirm the results obtained by the use of the gelatin filter. Owing to the

* 'Journ. Physiol.,' 1904, vol. 32; 'Proc.,' of November 12; 'Proc. Chem. Soc.,' 1905, vol. 21, p. 189, June 6.

† 'Zeit. Physiol. Chem.,' 1905, vol. 46, p. 136.

lack of experimental detail, Buchner and Antoni imagined that in our comparative experiments the concentration of glucose and of enzyme had not been kept constant, and ascribed part of the increase produced by boiled juice to the favourable effect of a diminution in the concentration of the sugar and of the alcohol, which is always present, by dilution with the added boiled juice. The details given above show that neither of these influences had any share in the effects observed by us.

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The Alcoholic Ferment of Yeast-juice. Part II.—*The Coferment of Yeast-juice.*

By ARTHUR HARDEN, D.Sc., Ph.D., and WILLIAM JOHN YOUNG, M.Sc.

(Communicated by Dr. C. J. Martin, F.R.S. Received June 14,—Read June 28, 1906.)

(From the Chemical Laboratory, Lister Institute.)

In a previous communication* it was shown that the fermentation of glucose by yeast-juice is dependent upon the presence of a dialysable substance which is not destroyed by heat. This substance is contained in the active yeast-juice prepared by disintegrating living yeast, and, therefore, most probably exists in the yeast cell side by side with the zymase.

The occurrence of an analogous activating substance has been described by Magnus† in the case of the lipase of the liver. He observed that the active juice of this organ became inactive when dialysed into water, but regained its activity when the dialysate or boiled liver juice was added. The term *coferment* was suggested by Bertrand‡ to denote substances of this kind, and he applied it in two instances—to the calcium salt, which he considered was necessary for the action of pectase on pecten substances, and to the manganese of laccase, which he supposed to be essential for the activity of this enzyme. Although not entirely satisfactory, this term may be provisionally applied to activating substances such as those present in liver lipase and yeast-juice, until further knowledge of their nature and function permits of a more rational terminology.

(1) *Preparation of the Inactive Residue from Yeast-juice in the Dry State.*

In the previous communication§ it was shown that when yeast-juice is filtered through a Martin gelatin filter, both the residue and the filtrate are incapable of fermenting sugar, whereas a mixture of the two produces a vigorous fermentation.

In carrying out the experiments which established this result, the residue left on the filter was always dissolved in water as soon as the filtration and washing were completed, and the activity of the solution was examined without delay. It has since been found possible to obtain the inactive

* 'Roy. Soc. Proc.,' B, vol. 77, 1906, p. 410.
† 'Zeit. Physiol. Chem.,' 1904, vol. 42, p. 149.
‡ 'Compt. Rend.,' 1897, vol. 124, p. 1032.
§ *Loc. cit.*

residue in the solid form, in which condition it retains its properties for a considerable time.

This is accomplished by spreading the sticky mass left on the filter over a large watch-glass, and exposing it over sulphuric acid in a vacuum. The residue dries up in a few hours to a brittle mass, which is converted by grinding into a light yellow powder.

Complete removal of the coferment is generally not effected by a single filtration, and the powder prepared as above is usually found to be slightly active. A completely inactive residue may, however, be obtained by redissolving in water and repeating the filtration and desiccation. The powder prepared in this way and dried over sulphuric acid in a vacuum for 15 hours only loses its potential activity slowly. The following examples show the original inactivity of the powder, its fermentative power in presence of boiled yeast-juice, and the loss of this power with lapse of time:—

I. 200 c.c. of yeast-juice were filtered in quantities of 50 c.c. through Martin filters and yielded 17·6 grammes of dry solid. This was dissolved in 50 c.c. of water and again filtered, yielding 9·2 grammes of dry solid residue.

The following experiments were then made at 26° in presence of toluene:—

1. March 8, 1906. One gramme of dry residue was dissolved in 15 c.c. of water and 1·5 grammes glucose added. No evolution of carbon dioxide was observed; 12 c.c. of boiled yeast-juice containing 1·2 grammes of glucose were then added. Fermentation at once commenced, and 108 c.c. of carbon dioxide were evolved in 20 hours, when the experiment was discontinued.

2. Two months later (May 18, 1906) a similar experiment was carried out, a sample of the same boiled yeast-juice being employed. As before, 1 gramme of the residue dissolved in sugar solution was quite inactive. On the addition of the boiled juice fermentation at once commenced; the rate was, however, only about one-third of that attained in the previous experiment, and fermentation ceased when only 42 c.c. of carbon dioxide had been evolved.

Although the fermentation in the first experiment was not continued to the end, the comparison shows clearly that the potential activity of the dry residue had greatly diminished.

II. In the case of another sample of dry residue, with which two similar experiments were made with the interval of a week, it was found that in the first experiment 364 c.c. of carbon dioxide were produced, and in the second 344 c.c., a difference of only about 5·5 per cent.

(2) *Disappearance of the Coferment from Yeast-juice during Fermentation and Autolysis.*

When a small amount of boiled yeast-juice is added to a solution of the inactive residue in 10-per-cent. glucose solution, fermentation commences and continues for a period which varies with the amount added. The cessation of fermentation appears in such a case to be due to some change in the coferment, since the addition of a further quantity of boiled yeast-juice again sets up fermentation, and if the quantity of boiled juice added on each occasion be small enough, this process can be repeated a third time.

The following experiments, made at 26° in presence of toluene, illustrate this point:—

1. One gramme of dry inactive residue was dissolved in 15 c.c. of 10-per-cent. glucose solution, and three quantities of 3 c.c. of boiled juice were then successively added, the fermentation being allowed to come to an end before each addition.

	Carbon dioxide evolved.
1st addition	8·2 c.c.
2nd „	6 „
3rd „	6 „

2. Two grammes of dry inactive residue were dissolved in 15 c.c. of 10-per-cent. glucose solution, and two quantities of 15 c.c. of a diluted boiled juice containing 10 per cent. of glucose were successively added.

	Carbon dioxide evolved.
1st addition.....	54 c.c.
2nd „	41·2 „

This phenomenon has been frequently observed in the course of experiments on the filtration of the juice through the Martin filter. The first residue obtained is generally slightly active, and produces a small amount of fermentation with glucose solution. The evolution of carbon dioxide, however, soon stops, but is again renewed when boiled yeast-juice is added. One instance of this was quoted in our previous communication,* and many more have been observed, some of which are tabulated below.

Carbon dioxide evolved from residue + glucose.	Boiled juice added.	Subsequent evolution of carbon dioxide.
c.c.	c.c.	c.c.
8·6	20	72
13·2	16	364·3
16·4	20	368·8

* *Loc. cit.*, p. 410.

The cessation of fermentation in any particular mixture of inactive residue and coferment may, therefore, be due to the disappearance either of ferment or coferment from the liquid. If the amount of coferment present be relatively small, it is the first to disappear, and fermentation can then only be renewed by the addition of a further quantity, whilst the addition of more ferment produces no effect. If, on the other hand, the amount of coferment be relatively large, the inverse is true; the ferment is the first to disappear, and fermentation can only be renewed by the addition of more ferment, a further quantity of coferment producing no effect. This is illustrated by the following experiment made at 26°:—

Two solutions were made up, each containing 0·5 gramme of dry inactive residue +10 c.c. of 10-per-cent. glucose solution and toluene, and to each of these 2 c.c. of boiled juice containing 10 per cent. glucose were added. As soon as fermentation had ceased, 0·5 gramme of dry residue in 10 c.c. of glucose solution was added to one solution (*a*), and 2 c.c. of boiled juice diluted to 10 c.c. with glucose solution to the other (*b*). Two other solutions were also prepared, each containing 0·4 gramme of the same dry inactive residue +10 c.c. of 10-per-cent. glucose solution + toluene, and 25 c.c. of the same boiled juice containing glucose were added to each.

As soon as fermentation had ceased, 0·4 gramme of residue dissolved in 10 c.c. of glucose solution was added to one (*c*), and 10 c.c. of boiled juice containing glucose to the other (*d*). The following were the results:—

	Boiled juice added.	Fermentation produced.	Subsequent addition.	Additional fermentation.
	c.c.	c.c.		c.c.
(*a*)	2	9·4	0·5 gr. residue	0
(*b*)	2	7·6	2 c.c. boiled juice	8·9
(*c*)	25	56·8	0·4 gr. residue	36·8
(*d*)	25	50	10 c.c. boiled juice	0

(3) *Rate of Disappearance of the Coferment from Yeast-juice.*

The following experiments were made to ascertain approximately the rate at which the coferment disappears from yeast-juice both in the absence and presence of added glucose. For this purpose a quantity of yeast-juice was preserved at 25° in presence of toluene, and samples were removed, boiled and filtered, at the commencement of the incubation and after various intervals. In one case a parallel experiment was made with yeast-juice to which 10 grammes of glucose per 100 c.c. had been added. The filtrates were then all rendered neutral to litmus and tested with equal quantities of an inactive

residue and glucose, care being taken to keep the concentration of glucose the same throughout.

Material digested.	Time of digestion.	Volume of filtrate taken.	Fermentation produced.
	days.	c.c.	c.c.
1. *a.* Yeast-juice alone	0	20	168·5
b. „	9	20	0
2. *a.* „	0	16	364·3
b. „	2	16	2·6
c. „	4	16	0
3. *a.* „	0	15	62·9
b. „	1	15	2·1
c. „	2	15	0
d. „	4	15	0
e. Yeast-juice + glucose.....	0	15	62·9
f. „	1	15	15·6
g. „	2	15	4·5
h. „	4	15	8·3

In two cases in which a negative result was obtained (1*b* and 2*c*) an equal volume of the same fresh boiled juice was added and in both cases a vigorous fermentation was produced, thus proving that the autolysed juice had not exerted any specific detrimental effect on the ferment:—

	Fresh boiled juice added.	Carbon dioxide evolved.
1. *b*.....	20 c.c.	112 c.c.
2. *c*.....	16 „	297 „

It appears from this that the coferment disappears from the juice less rapidly in the presence of glucose than in its absence. In yeast-juice to which no addition of glucose has been made the coferment usually disappears at 26° in about 48 hours; in the presence of 10 per cent. of glucose coferment was still present at the end of four days, although only in a small amount. (The observation that the fermentation after four days' incubation is somewhat greater than that given after two days is probably to be explained by the presence of a greater amount of phosphate in the juice which had been digested for the longer period.)

4. *Soluble Phosphates do not Render the Inactive Residue Capable of Fermenting Glucose.*

In view of the fact that soluble phosphates, as described in the previous communication, exert a remarkable effect on the fermentation of glucose by yeast-juice, experiments were made to ascertain whether the addition of a soluble phosphate to a solution of the inactive residue in glucose is sufficient

to set up fermentation. All the attempts hitherto made to effect this have yielded entirely negative results, although both the kind of phosphate and the amount added have been varied.

Dipotassium hydrogen phosphate, a mixture of this with the dihydrogen phosphate, disodium hydrogen phosphate, diammonium hydrogen phosphate, microsmic salt, and a mixed phosphate of potassium and magnesium obtained by boiling a solution of potassium dihydrogen phosphate with magnesium carbonate, were employed, all of which are capable of producing the characteristic effect of phosphates on the fermentation of glucose by yeast-juice.

Although these substances did not set up fermentation when added to an inactive filtered residue and glucose, they did not affect the potential activity of the residue. This is evident from the fact that the subsequent addition of boiled juice produced an immediate fermentation.

In every case the solution of the phosphate was saturated with carbon dioxide at 26° and added to the solution of the inactive residue in glucose solution also saturated with carbon dioxide at 26°, and in no case was any evolution of gas observed after the cessation of the slight disturbance which inevitably occurs when the solutions are mixed. The phosphate solutions were all of 3/10 molar strength, with the exception of the mixed phosphate of magnesium and potassium, 5 c.c. of which yielded 0·1594 gramme $Mg_2P_2O_7$.

In those cases in which the residue employed was slightly active, incubation was continued until all fermentation had ceased before the phosphate solution was added, and the amount of carbon dioxide evolved in this preliminary period is given in the table. The amount of glucose, both in the solution of the residue and in that of the phosphate, was throughout 10 grammes to 100.

	Amount of residue.	Preliminary fermentation.	Phosphate added.	Carbon dioxide produced.	Subsequent evolution of carbon dioxide after the addition of boiled juice.
1	25 c.c.	41·2	2·5 c.c. KMg phosphate ...	0	18·4
2	25 „	47·7	5 „ „ ...	0	—
3	25 „	0·6	3 „ K_2HPO_4	0	268·8
4	1·6 gr.	21·5	3 „ KMg phosphate ...	0	159·2
5	1·2 „	10·7	5 „ „ ...	0	—
6	0·5 „	0	2 „ mixture of K_2HPO_4 and KH_2PO_4	0	3 c.c. per hour
7	0·5 „	0	2 „ $(NH_4)_2HPO_4$	0	4·2 „ „
8	0·5 „	0	2 „ $(NH_4)NaHPO_4$ ...	0	3·8 „ „
9	0·5 „	0	2 „ Na_2HPO_4	0	3·6 „ „

The foregoing conclusion is confirmed by the observation, made in the

experiments on the disappearance of the coferment from yeast-juice, that boiled autolysed juice does not set up fermentation in a mixture of the inactive residue with glucose, although it itself contains a large amount of phosphate precipitable by magnesia mixture. The following numbers were obtained by the analysis of three specimens of boiled autolysed juice employed in those experiments, two of which were quite inactive, whilst the other only produced a fermentation of 2·6 c.c.

No. of experiment.	Volume of juice.	Carbon dioxide evolved.	Phosphate present in grammes of $Mg_2P_2O_7$.
	c.c.	c.c.	
1. *b*	20	0	0·3400
2. *b*	16	2·6	0·3011
3. *c*	15	0	0·1893

These experiments throw no light on the actual chemical nature of the coferment, but show that most probably it does not consist of a phosphate precipitable by yeast-juice. They also indicate that substances, which, like phosphates, increase the total fermentation produced by yeast-juice, are not necessarily capable of setting up fermentation when added to a mixture of inactive residue and glucose.

Further experiments are in progress with the hope of obtaining more information on these points.

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Chemical Action of Bacillus lactis aerogenes (Escherich) *on Glucose and Mannitol: Production of* 2 : 3-*Butyleneglycol and Acetylmethylcarbinol.*

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(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—Read February 1, 1906.)

(From the Chemical Laboratory, Lister Institute.)

I. *Action of B. lactis aerogenes on Glucose.*

In the course of an investigation on the chemical action on glucose of the lactose-fermenting bacteria of fæces* it was found that, whereas *B. coli communis* yields, with glucose, acetic acid and alcohol in approximately equal molecular proportions, *B. lactis aerogenes* produces a much smaller amount of acetic acid, relatively both to the alcohol and to the sugar fermented. It was, moreover, previously known that *B. lactis aerogenes* produces a greater volume of gas, containing a larger proportion of carbon dioxide than does *B. coli communis*.† These facts indicate that the fermentation of glucose by this organism is of a different type from that brought about by the *B. coli communis*.‡ A detailed examination of the products was therefore made. For this purpose the organism was grown anaerobically in a medium containing 1 per cent. of Witte peptone and 2 per cent. of pure glucose in the presence of chalk, the method of collecting the gases evolved and of examining the products being the same as that previously employed in the investigation of the action of *B. coli communis* on glucose.§

All the substances produced by *B. coli communis* from glucose were again found, viz., lactic acid, acetic acid, succinic acid, formic acid, ethyl alcohol, carbon dioxide, and hydrogen. A quantitative estimation, however, revealed the fact that only about two-thirds of the carbon of the glucose was thus accounted for. This is shown in the following tables, which embody the results of three separate estimations (Cols. 1, 2, 3), Table I giving the actual percentages by weight on the sugar fermented of the various substances produced, and Table II the number of carbon atoms per molecule of sugar decomposed represented by each product. The results of a typical

* Harden, 'Journ. of Hygiene,' 1905, vol. 5, p. 488.

† Theobald Smith, 'Centralbl. f. Bakteriol.,' 1895, vol. 18, pp. 1, 494, 589.

‡ Harden, 'Trans. Chem. Soc.,' 1901, p. 601.

§ Harden, *loc. cit.*

fermentation of glucose by *B. coli communis* are added (Col. 4) for the sake of comparison :—

Table I.

	1.	2.	3.	4.
Alcohol	17·1	16·4	18·2	12·85
Acetic acid	5·1	4·2	8·6	18·84
Lactic acid	5·5	4·7	9·1	31·90
Succinic acid	2·4	3·1	4·5	5·20
Formic acid	1·0	0·75	1·7	0·0
Carbon dioxide	38·0	—	35·2	18·09
Carbon dioxide, c.c. per gramme	198·3	—	178·5	91·8
Hydrogen, c.c. per gramme	82·4	—	92·4	110
Ratio H_2/CO_2	0·42	—	0·52	1·19
Percentage excess of l-lactic acid	50·0	66·0	86·0	Not determined

Table II.

	1.	2.	3.	4.
Alcohol	1·34	1·28	1·43	1·01
Acetic acid	0·31	0·25	0·52	1·13
Lactic acid	0·33	0·28	0·55	1·91
Succinic acid	0·15	0·19	0·27	0·32
Formic acid	0·04	0·02	0·07	0·00
Carbon dioxide	1·60	—	1·44	0·74
Total	3·77	—	4·28	5·11
Hydrogen, atoms per molecule	1·33	—	1·50	1·77

It will be observed that the ratio of hydrogen to carbon dioxide by volume is about 0·5 to 1, whilst these gases are produced by *B. coli communis* in approximately equal volumes. Theobald Smith,* using an ordinary fermentation tube, gives the characteristic ratio for *B. coli communis* as $H_2/CO_2 = 2:1$ and for *B. lactis aerogenes* $H_2/CO_2 = 1:1$. This difference is due to the solubility of the carbon dioxide in the liquid medium, and it must be remembered that while Smith's ratios give a perfectly satisfactory working test for the discrimination of the organisms, they do not represent the actual volumes or ratios of the gases produced.

Further examination of the fermentation products revealed the fact that

* *Loc. cit.*

no other acids had been formed, and search was therefore made for compounds of a different type. It was previously suggested that the deficiency of carbon observed in the fermentation produced by *B. coli communis,* amounting to only 0·25 to 0·9 of an atomic proportion of carbon, might possibly be due to the presence of reduction products of sugar, and compounds of this kind were therefore sought.

It was found that when the neutral liquid, containing the products of fermentation along with peptone, was evaporated to dryness at 55° under diminished pressure and extracted with alcohol, a solution was obtained which yielded on fractionation a colourless liquid boiling at 181° to 183° (corr.) at 760 mm. pressure. The yield was very small, only amounting to about 1 gramme per litre of medium containing 20 grammes of glucose, but it was found possible to increase the yield by employing a medium containing 5 per cent. of glucose, and in this way 8 grammes of the new substance, containing 52·8 per cent. of carbon, were obtained per litre of medium containing 50 grammes of sugar. This only accounts for about two-thirds of the missing carbon, and a rough estimate of the amount lost during the process of distillation and extraction was, therefore, made by dissolving 8 grammes of the material in 500 c.c. of a medium containing 5 grammes of Witte peptone, 6 grammes of calcium lactate and 6·5 grammes of alcohol and then extracting it in the manner described above. Only 5·2 grammes were recovered, the loss per 500 c.c. being therefore about 2·8 grammes and the loss per litre about 5·6 grammes. This brings the total amount produced from 50 grammes of glucose to about 13·6 grammes, slightly in excess of that required. It is hoped that the actual yield may be increased by a careful fractionation of the fermentation products.

The new product is apparently a mixture, and it has not yet been found possible to separate and identify all the components, so that the following must be taken as only a preliminary account of the substance.

It boils at 181° to 183° (corr.), and solidifies in the cold to a transparent mass which melts indefinitely at about 28°. It is optically active, the value for $[\alpha]_D$ for different preparations varying from 0·46 to 0·71. The composition of the substance dried by quicklime is approximately that of a butyleneglycol, but the percentage of carbon is about 0·6 too low. It does not reduce Fehling's solution either in the cold or on heating. That this substance contains a large proportion of 2 : 3-butyleneglycol, $CH_3.CH(OH).CH(OH).CH_3$, is shown by the following facts :—

1. When the liquid is heated with phenylisocyanate dissolved in anhydrous ether, combination occurs and a mixture of urethanes is produced. The fraction of these which is least soluble in alcohol comprises about 90 per cent.

of the whole amount and has the composition of the diphenylurethane of butyleneglycol ($C_4H_{10}O_2.2C_6H_5NCO$):—

Analysis.

	Found.	Calculated.
C	65·79	65·85
H	6·21	6·09
N	8·57	8·53

It is sparingly soluble in cold alcohol, ether and benzene, crystallises in rosettes of needles and melts at 197° to 198° (uncorr.). When the urethane is boiled with baryta water or caustic soda solution it is decomposed and yields a glycol boiling at about the same temperature as the original material. This glycol has, however, not yet been isolated in the perfectly pure and dry state. A monourethane, $C_4H_{10}O_2C_6H_5NCO$, has also been prepared which is somewhat more soluble in cold alcohol than the diurethane, and crystallises in needles, melting at 100°:—

Analysis.

	Found.	Calculated.
N	6·89	6·65

2. Both the crude glycol and that recovered from the diurethane are converted by oxidation with bromine in the light* into diacetyl, $CH_3.CO.CO.CH_3$ which was recognised by its extremely characteristic appearance and smell, and by the formation of a phenylosazone melting at 242° to 242°·8 (uncorr.).† The formation of this substance shows conclusively that 2 : 3-butyleneglycol must be present in the fermentation product.

Detection of Acetylmethylcarbinol among the Fermentation Products.—It was further found that the distillate from the liquid in which the organism was grown reduced Fehling's solution in the cold and gave with phenylhydrazine the osazone of diacetyl, melting at 243°. These properties point to the presence in the distillate of acetylmethylcarbinol, $CH_3.CO.CH(OH).CH_3$, which has previously been detected in this way by Grimbert‡ and by Desmots§ in the products of the fermentation of glucose by several bacteria: *B. tartricus, B. mesentericus vulgatus, B. fuscus, B. flavus, B. niger, B. ruber, B. subtilis,* and *Tyrothrix tenuis.* It has also been found in vinegar.

This compound appears only to be formed in very small amount. Since it

* v. Pechmann, 'Ber.,' 1890, vol. 23, p. 2427.
† v. Pechmann, 'Ber.,' 1888, vol. 21, p. 2754.
‡ 'Compt. Rend.,' 1901, vol. 132, p. 706.
§ 'Compt. Rend.,' 1904, vol. 138, p. 581.

is likewise converted into diacetyl by oxidation with bromine in the light, it is important to notice that the glycol used for conversion into diacetyl, as described above, was quite free from any substance capable of reducing Fehling's solution, and yielded a relatively large amount of diacetyl.

II. *Action of B. lactis aerogenes on Mannitol.*

A quantitative examination of the products of fermentation of mannitol by *B. lactis aerogenes* showed that in this case also the action differed from that produced by *B. coli communis*, but that the deficit of carbon was only one-half of that found for glucose. This is shown in the following tables: Table III giving the percentages and Table IV the number of carbon atoms per molecule of mannitol represented by the products in two experiments (Cols. 1 and 2). As before, the products obtained by the action of *B. coli communis* are also given for the sake of comparison (Col. 3):—

Table III.

	1.	2.	3.
Alcohol	32·5	32·5	28·1
Acetic acid	2·5	2·1	9·5
Lactic acid	8·6	8·6	18·6
Succinic acid	3·2	2·8	8·9
Formic acid	1·5	1·6	3·0
Carbon dioxide	35·5	35·5	28·44
Carbon dioxide, c.c. per gramme	180·3	180·3	143·0
Hydrogen, c.c. per gramme	138·3	143·6	167·0
Ratio H_2/CO_2	0·77	0·79	1·18
Percentage excess of 1-lactic acid	65·0	56·0	79·0

Table IV.

	1.	2.	3.
Alcohol	2·57	2·57	2·22
Acetic acid	0·15	0·12	0·58
Lactic acid	0·52	0·52	1·13
Succinic acid	0·20	0·17	0·55
Formic acid	0·06	0·065	0·12
Carbon dioxide	1·47	1·47	1·16
Total	4·97	4·91	5·76
H atoms per molecule glucose	2·26	2·34	2·7

Further examination has shown that in this case as in that of glucose, both acetylmethylcarbinol and a glycol are produced, but both in much less quantity. The amount of crude glycol actually isolated from the products of fermentation of 50 grammes of mannitol was only 0·75 gramme. Since, however, the loss in isolating may be roughly taken as about 5 grammes, this is approximately the yield which would be expected if 6 to 7 grammes were formed. The nature of these products and their quantitative estimation, as well as the study of their optical properties, is still under investigation, and search is also being made for these and similar substances among the fermentation products of other bacteria.

General Considerations.

The production of so large a proportion of 2 : 3-butyleneglycol in these experiments affords clear proof that this substance is derived from the glucose. The interesting question as to the mode of its production from the glucose or mannitol molecule will be best deferred until a more complete examination of the products, and especially of their optical relations, has been made. The close constitutional relation between the glycol and lactic acid, and the readiness with which its oxidation product—diacetyl—passes into an aromatic compound are also points of great interest. It may, however, be noted that the comparison of the fermentation products of *B. coli communis* and *B. lactis aerogenes* shows, firstly, that the alcohol produced by the latter organism is slightly greater in amount than that due to the former, and, secondly, that it is at the expense of that part of the molecule which in the *B. coli* fermentation yields acetic acid and lactic acid, that the *B. lactis aerogenes* forms the new products.

It may further be observed that both these bacteria produce twice as much alcohol from mannitol as from glucose, a fact which tends to confirm the suggestion previously made,* that the formation of alcohol in these reactions is related to the presence of the terminal $CH_2(OH).CH(OH)$ group, which occurs twice in the molecule of mannitol and only once in that of glucose.

A substance of the composition of butyleneglycol has previously been isolated from the products of fermentation of sugar by yeast,† and was also found in wine‡ and in brandy.§ This substance, boiled at 178° to 179°, yielded a diacetin boiling at 192° to 193°, and was considered to be identical

* Harden, 'Trans. Chem. Soc.,' 1901, p. 601.

† Claudon and Morin, 'Compt. Rend.,' 1887, vol. 104, p. 1109; Henninger and Sanson, 'Compt. Rend.,' 1888, vol. 106, p. 208.

‡ Henninger, 'Compt. Rend.,' 1882, vol. 95, p. 94.

§ Morin, 'Compt. Rend.,' 1887, vol. 105, p. 1019.

with the synthetical isobutyleneglycol of Nevolé,* which boils at 176° to 178°. The yield obtained from sugar was, however, very small, and only amounted to about 0·2 per cent. after allowing for the losses involved in the extraction of the compound.

In view of the properties of the crude glycol described above, it would seem advisable to re-examine Henninger's glycol, the constitution of which was not experimentally examined.

* 'Compt. Rend.,' 1876, vol. 83, p. 65.

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TRYPSIN AND ANTITRYPSIN

By S. G. HEDIN

From the Lister Institute of Preventive Medicine

(*Received August 10th*, 1906)

In a previous paper[1] I have given an account of experiments on the antitryptic action of serum-albumin, the main results of which were :—

(1) If the trypsin and the antibody are mixed before they are added to the substrate, then the neutralising effect of the antibody is larger than if they are added separately. (2) The effect increases up to a certain point, when the trypsin and the antibody are kept in contact before the addition of the substrate. (3) The maximum effect of the antibody is larger the higher the temperature. The effect corresponding to a certain temperature increases if the temperature is raised, but remains the same if the temperature is lowered.

The continuation of these experiments is given in the following paper. The estimation of the digesting power was carried out by precipitating the samples to be compared with equal volumes of tannic acid solution and determining the nitrogen in equal volumes of the filtrates[2]. Therefore the figures given represent the number of cubic centimetres of decinormal acid, corresponding to the nitrogen in equal volumes of the tannic acid filtrate. In all experiments the antibody was allowed to act upon the trypsin before the substrate (casein) was added. It may be pointed out that the proteid in the antibody solution (the serumalbumin dialysed free from salt) is practically indigestible, unless a very large excess of trypsin is used. An amount of trypsin, which with an excess of casein would have given the effect 90, gave with 10 c.c. of antibody the effect 0·2.

1. *Journ. of Physiol.*, Vol. XXXII, p. 390, 1905.

2. The tannic acid solution contained in two litres: 200 grs. tannic acid, 100 c.c. glacial acetic acid, 50 grs. sodium chloride, and 50 grs. sodium acetate.

The Influence of the Concentration of the Trypsin-Antitrypsin Mixture on the Amount of Trypsin Neutralised.

That concentration effects no material influence is borne out by the following experiments :—

Experiment I.—In this experiment the ratio between the concentrations to be compared was 2 : 1. Two sets of specimens containing the one (A) 50 c.c. tryps. + 10 c.c. (antib. + H_2O), and the other (B) 50 c.c. tryps. + 10 c.c. (antib. + H_2O) + 60 c.c. H_2O, were prepared and kept at 21°. After the intervals of time recorded 10 c.c. of the specimens of set A, together with 10 c.c. of water, were added to 50 c.c. of casein solution. Of the specimens of set B, 20 c.c. were added to the same amount of casein. After 24 hours at 37°, the nitrogen was determined in equal volumes of the tannic acid filtrates.

		20 min.	4 hours.	24 hours.
0·625 c.c. antib.	A	36·05	36·25	—
	B	36·35	35·9	—
1·25 ,,	A	32·65	33·2	—
	B	32·45	32·65	—
2·5 c.c. ,,	A	20·0	20·1	—
,,	B	19·55	19·2	—
5 c.c. ,,	A	8·2	6·25	6·0
	B	8·4	6·75	5·5
10 c.c. ,,	A	5·35	4·55	4·4
	B	5·4	4·8	4·15

In the specimens with 0·625, 1·25 and 2·5 c.c. of antibody, the final state of equilibrium was reached in 20 min., but not in those with larger amounts of antibody. In no case can any influence of the concentration be observed.

Experiment II.—Ratio between concentrations 4 · 1. Mixtures kept at 37°.

(A) 20 tr. + 100 antib.
(B) 20 tr. + 100 antib. + 360 H_2O.

Of A, 25 c.c., and of B, 100 c.c. were taken for digestion as above.

	5 min.	30 min.	1 Hour
A	4·6	3·8	3·75
B	4·85	4·4	4·2

Of the same agents another two mixtures were prepared :

(A) 20 tr. + 1 antib.

(B) 20 tr. + 1 antib. + 63 H_2O.

and 5 resp. 20 c.c. taken for digestion.

	5 min.	30 min.
A	32·95	32·45
B	32·4	32·05

It must be borne in mind that Experiments 1 and 2 were carried out with such small amounts of casein that the results are not proportional to the amounts of active trypsin present,[1] and therefore, the results do not allow of any conclusions as to the relative amounts of trypsin neutralised by different amounts of antibody.

Can any of the Agents (Trypsin or Antibody) be completely Neutralised ?

My investigations on this subject show, that when little antibody is used, then the antibody can be completely neutralised, as proved by the fact that fresh trypsin added develops its full activity. In the cases tried more than three-quarters of the whole amount of trypsin could be neutralised without leaving any free antibody. On the other hand, if much antibody is used, then some antibody remains free, the fluid showing strongly antitryptic action upon added trypsin ; at the same time the fluid manifests some tryptic activity even after 24 hours in presence of very large amounts of antibody. In short, *the antibody can be completely neutralised by using a sufficient amount of trypsin, whilst it is impossible to neutralise all trypsin by an excess of antibody.*

Two series of experiments bearing on this subject are recorded below. In these, such an excess of casein was used that the tryptic effect was proportional to the active trypsin present.[2] This was proved to be the case by the following experiment, where the effects of 2·5 and 5 c.c. of trypsin were found to be 25·7 resp. 51·7. Therefore all figures less than 51·7 are inside the range of proportionality.

1. Hedin, 'Observations on the action of trypsin,' *Journ. of Physiol.*, Vol. XXXII, p. 468, 1905.

2. *Journ. of Physiol.*, Vol. XXXII, pp. 471-474, 1905.

Experiment III.—Each specimen contained 300 c.c. casein + 10 c.c. (tryps. + H_2O) + 1 c.c. (antib. + H_2O). The trypsin or, in case the trypsin was added in two lots, the first lot of it, the antibody and the water were kept for two hours at 37°, then the second lot of trypsin was added, and all specimens were kept for another hour at 37° before the casein was added. The digestion lasted for 24 hours. 150 c.c. tannin acid solution and 300 c.c. filtrate were taken for each analysis.

1.	2·5 c.c. tryps. without antibody	25·7
2.	5 c.c. „ „	51·7
3.	5 c.c. „ + 0·125 c.c. antib.	21·55
4.	5 c.c. „ + „ + 2·5 c.c. tryps ...	47·35
5.	5 c.c. „ + 0·25 c.c. antib.	11·95
6.	5 c.c. „ + „ + 2·5 c.c. tryps. ...	36·9

The effect of an addition of 2·5 c.c. of trypsin is 47·35 — 21·55 = 25·8 in Specimen 4, and 36·9 — 11·95 = 24·95 in Specimen 6, as against 25·7 in Specimen 1 without antibody, showing that in both cases the antibody was completely engaged before the second lot of trypsin was added.

Experiment IV was carried out like Experiment III, only with another enzyme.

1.	5 tryps. without antibody	35·85
2.	„ + 0·25 antib.	8
3.	„ „ + 5 tryps.	43·25
4.	„ + 0·5 antib.	6·6
5.	„ „ + 5 tryps.	17·75
6.	„ + 0·75 antib.	6·65
7.	„ „ + 5 tryps.	8·95
8.	„ + 1 antib.	6·55
9.	„ „ + 5 tryps.	8·75

Specimen 3 shows that the addition of 5 c.c. of trypsin to Specimen 2, gives the effect 43·25 — 8 = 35·25, or practically the same effect as 5 tryps. without the presence of antibody (35·85 in Specimen 1). On the other hand, Specimens 4-9, which all contained more antibody than 0·25 c.c., show that the effect of the second lot of trypsin was not nearly the same as without antibody. Therefore, in Specimens 5, 7, 9, there was active antibody present, when the second lot of trypsin was added

Experiment V was carried out with large amounts of antibody and long time of interaction of the agents. The specimens contained :

100 c.c. cas. + 12 c.c. (tryps. + H_2O) + 3 c.c. (antib. + H_2O).

Trypsin and antibody were kept at 37° for 24 hours, then the second lot of trypsin and the casein were added. After 24 hours' digestion, 50 c.c. tannic acid solution were added and 100 c.c. filtrate were taken for analysis.

1.	2 tryps. without antibody	9·0
	Therefore 10 c.c tryps. corresponds to	45·0
2.	10 tryps. + 1 antibody	2·1
3.	„ „ + 2 tryps.	4·9
4.	„ + 2 antib....	1·95
5.	„ „ + 2 tryps.	2·55
6.	„ + 3 antib.	1·95
7.	„ „ + 2 tryps.	2·45

The figures obtained indicate that in Specimens 2, 4, 6 there was free trypsin and free antibody present at the same time.

The Amount of Trypsin Neutralised by a certain Amount of Antibody

Experiments bearing on this point show, that when different amounts of antibody are made to act upon the same amount of trypsin, then *the effect of a small amount is always greater per unit of antibody.* In the experiments such an amount of casein was used that the results obtained were proportional to the active amount of trypsin present.

Experiment VI.—This experiment is the same as Experiment III, only with the results differently arranged and with an additional set of specimens containing 10 c.c. of trypsin.

1.	5 tryps. + 0 antib. ...	51·7		4.	10 tryps. + 0 antib. ...	103·4[1]
2.	„ + 0·125 „ ...	21·55		5.	„ + 0·25 „ ...	44·1
3.	„ + 0·25 „ ...	11·95		6.	„ + 0·5 „ ...	25·95

According to Experiment III, the Specimens 2 and 3 contained no free antibody. The same, therefore, must have been the case with 5 and 6, which had the double amounts of agents and gave approximately the double effect. Therefore, on complete binding, the

1. Since this figure lies outside the range of proportionality, it has been calculated by doubling the effect of 5 c.c. of trypsin.

neutralising effect of 0·125 c.c. of antibody is found to be 30·15 from 1 and 2, and 9·6 from 2 and 3, and for 0·25 c.c. of antibody one obtains the effect 59·3 from 4 and 5, and 18·15 from 5 and 6.

Experiment VII is the same as Experiment IV, with an additional set of specimens.

5 tryps.	+ 0	antib.	...	35·85	10 tryps	+ 0	antib.	...	71·7[1]
„	+ 0·25	„	...	8	„	+ 0·25	„	...	41·95
„	+ 0·5	„	...	6·6	„	+ 0·5	„	...	15·65
„	+ 0·75	„	...	6·65	„	+ 0·75	„	...	9·6
„	+ 1	„	...	6·55	„	+ 1	„	...	9·5

The figures obtained illustrate the above rule without any further explanation. In both sets, with large amounts of antibody, a state of neutralisation was arrived at which was not altered on addition of more antibody.

Destruction of the Antibody

The antitryptic properties of the serumalbumin are destroyed on boiling, and even on heating to a lower temperature, as shown by Cathcart.[2] At bloodheat it is readily destroyed by 0·2% acetic acid, as shown by following experiments.

The antibody was kept with 0·2% acetic acid at 37°. After intervals of time recorded, samples were taken, from which the acid was removed by dialysis, whereupon the neutralising effect upon trypsin was tried. After the antibody had been acting upon the trypsin for 2 hours at 37° the amount of trypsin left free was determined with 200 c.c. of casein solution. The results were :—

Without antibody	55·5
With 5 c.c. intact antibody	2·1
„ „ treated with acetic acid for 3 hours	45·9
„ „ „ „ 6 „	48·7

In another experiment, the same antibody was treated with acetic acid for 8 hours :—

Without antibody	35·95
With antibody treated with acetic acid for 8 hours ...	35·45

1. Since this figure lies outside the range of proportionality, it has been calculated by doubling the effect of 5 c.c. of trypsin.

2. *Journ. of Physiol.*, Vol. XXXI, p. 495, 1904.

Therefore the antibody is practically destroyed by 0·2% acetic acid during 8 hours at 37°.

Having thus found that acetic acid readily destroys the antibody and knowing that trypsin is only very slowly attacked by this acid, I thought it important to try whether, in a mixture of trypsin and antibody, which has been kept till the antibody has reached its maximum of effect, the trypsin can be rendered active again by treatment with 0·2% acetic acid at 37°. With this view, I have carried out several experiments, and I have been able to recover the trypsin in none of them. The figures obtained in one of them were as follows :

Without antibody	70
0 hours with acetic acid	3·6
2 ,, ,, ...	2·9
4 ,, ,, ...	2·5
8 ,, ,, ...	2·2

The slight fall in the activity of the mixture might depend upon the destructive influence of the acetic acid upon the trypsin not neutralised by the antibody[1].

Since some time is always required before all of the antibody is destroyed by the acetic acid, the antibody exercises some neutralising effect even in presence of acetic acid.

Discussion of the Results

It is generally assumed that enzymes, previous to their action, are in some way attached to the substrate they are able to act upon. On the other hand, antibodies are supposed to form a kind of combination with the substances they neutralise, thus preventing them from being attached to the substrate. My observations, that the amount of trypsin neutralised is larger when the antibody is allowed to act upon the trypsin in absence of a proteid than in presence of the same (p. 474), are quite consistent with these views.

1. With regard to this experiment it may be noted that Morgenroth on treating the compound cobrahaemolysin-antibody and cobraneurotoxin-antibody with HCl was able to recover all of the haemolysin and 50 per cent. of the neurotoxin, *Berl. Klin. Wochenschr*, 1905, No. 50, and 'Weitere Beiträge zur Kenntniss der Schlangengifte und ihrer Antitoxine'; *Arbeiten aus dem Pathol. Inst. zu Berlin*, 1906.

From the fact that the neutralising effect of the antibody is independent of the dilution (p. 475), one must conclude that the compound which might be formed is not subject to decomposition with water (hydrolysis). There are two other reasons which make me believe that the compound, once formed, does not give off any active trypsin to water. These are :—

1.—The antibody neutralises more trypsin the higher the temperature. Nevertheless, if a mixture of trypsin and antibody is kept at a certain temperature till a constant state of neutralisation has been reached, then no trypsin is liberated on lowering the temperature (p. 474).

2.—The antibody is readily destroyed by the action of weak acetic acid at 37°. If a mixture of trypsin and antibody, having been kept at 37° till no more trypsin is neutralised, is digested with weak acetic acid, then no trypsin is rendered active (p. 480).

It must, however, be borne in mind, that the way in which my experiments have been carried out does not exclude the possibility that, after the addition of casein, some trypsin might be transferred from the antibody to the casein. If the antibody readily gave off the trypsin, then the distribution of the trypsin would become the same as if the casein had been present from the very beginning. The fact that this is not the case shows that the trypsin, when once attached to the antibody, is not readily given off to casein. Nevertheless, it cannot be entirely excluded that some change may take place, and a transport of trypsin from the antibody to the casein might, perhaps, account for the fact that it has been found impossible completely to neutralise trypsin by means of the antibody (p. 476).

Since trypsin and its antibody undoubtedly, in more than one respect, behave in a way similar to some toxins and their antitoxins, it might be useful to consider the question, whether the theories put forth for the explanation of the relationship between the latter might apply to the trypsin and its antibody.

According to Ehrlich's theory,[1] the compound toxin-antitoxin is

1. *Fortschritte d. Med.*, 1897; *Werthbestimmung des Diphteriheilserums*, 1897.

merely a chemical one, like the combination between a strong base and a strong acid. This theory, therefore, involves that the two constituents should combine till one of them is completely bound up and that they by proper means can be separated again. Moreover, Ehrlich assumes that some toxins (*e.g.* diphteriatoxin) are made up of constituents of different toxicity and different avidity to the antibody. By aid of this theory it might be explained that a large amount of trypsin can be neutralised without leaving any antibody free, but the fact that on further addition of antibody the fluid may contain free trypsin and free antibody at the same time this theory does not account for, unless one assumes that trypsin is made up of different constituents. Furthermore, the fact that I have not been able to separate the two constituents of the trypsin-antitrypsin compound is not in favour of Ehrlich's theory.

Arrhenius and Madsen[1] assume that toxin and antitoxin behave like a weak base and a weak acid. Therefore, the compound is subject to hydrolysis, and there always exists some free toxin and some free antitoxin at the same time. Moreover, their theory requires that the compound should be reversible, *i.e.*, the equilibrium ought to be the same under the same conditions, no matter in which way they have been arrived at. This theory seems to be inconsistent with the fact that I have been able to neutralise more than three-quarters of the trypsin without any antibody remaining free in the fluid. In addition, the compound has not been found to be reversible in my experiments (p. 481).

Bordet has suggested that the antibodies be capable of combining with varying amounts of toxin, and consequently he holds that the combination toxin-antitoxin be not a chemical one. He assumes that the toxin is taken up by the antitoxin, very much as a dye is by a tissue. This view takes into account the fact that the agents, being colloids, may, perhaps, not follow the laws of genuine solutions.[2] This view has more recently been advanced by Nernst[3] and by Craw.[4]

1. *Festskrift ved invielsen af statens seruminstitut*, Copenhagen, p. 56, 1902.
2. *Ann. Inst. Past.*, Vol. XVII, p. 161, 1903.
3. *Zeitschr. physik. Chem.*, Vol. XLVII, p. 54, 1904.
4. *Proc. Roy. Soc.*, Vol. LXXVI, B, p. 179, 1905.

Trypsin and antitrypsin, being colloids, do not form any real solutions with water, but only suspensions of larger or smaller particles. Since very little is known as to the ways in which suspended particles might act upon each other, I have tried to throw some light upon this subject by investigating how charcoal acts upon trypsin, thus substituting charcoal for the tryptic antibody in my above experiments. The results are laid down in the following paper and the further discussion of the subject must be postponed till the account of these experiments has been given.

The results of my previous investigations on trypsin and antitrypsin are given on p. 474. The conclusions of the investigations in this paper are as follows :—

1.—The amount of trypsin neutralised by the antibody is independent of the dilution.

2.—The antibody can be completely saturated by using a sufficient amount of trypsin ; on the other hand it has been found impossible under the conditions used, completely to neutralise trypsin by an excess of antibody.

3.—The amount of trypsin neutralised by a certain amount of antibody is not constant, a small amount of antibody neutralising relatively more trypsin than a larger.

4.—The antibody can be completely destroyed by 0·1—0·2% of acetic acid at 37° for about 8 hours.

5.—When trypsin has been neutralised by the antibody, it has been found impossible by the means tried to render it active again.

FESTSCHRIFT FÜR OLOF HAMMARSTEN. VI.

AN EXPLANATION OF THE INFLUENCE OF ACID AND ALKALI ON THE AUTOLYSIS OF ORGANS

BY

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E. Salkowski in 1890 published investigations showing that liver, muscles, and suprarenal glands, when kept at 37° with antiseptics, undergo a change more or less similar to the digestion of proteids in the alimentary canal[1]. Similar observations had been previously made by Salomon[2] and by Schmiedeberg[3]. The investigations on this subject have been carried further by Salkowski's pupils, of whom Schwiening found that Na_2CO_3 checks the process[4] and Biondi observed a favourable influence of HCl upon the autodigestion of the liver[5]. Hedin and Rowland found that the proteolytic digestion of juice pressed from different organs went considerably faster in the presence of a weak acid (acetic acid, lactic acid) than in an alkaline medium[6]. The principal organs for which this rule was found to hold good, were spleen, liver, kidneys, lymphatic glands. Similar observations have been made by Hildebrand[7], Baer and Loeb[8], Wiener[9], and Schryver[10]. Investigating the proteolytic properties of the spleen of the ox, I found that this organ in addition to an enzyme acting only or principally in an acid medium (β-protease) contains an enzyme acting principally in an alkaline medium (α-protease)[11]. This enzyme, however, could not be properly

[1] Zeitschr. f. Klin. Med., 1890, Suppl.
[2] Arch. f. Anat. u. Physiol., 1881, p. 361.
[3] Arch. f. exp. Pathol. u. Pharm., 14, p. 379.
[4] Virchows Arch., 136, p. 444, 1894.
[5] Ibid., 144, p. 373, 1896.
[6] Zeitschr. f. physiol. Chem., 32, p. 341, 531, 1901.
[7] Hofmeisters Beiträge, 5, p. 463, 1904.
[8] Arch. f. exp. Pathol. u. Pharm., 51, p. 1, 1905.
[9] Zentralbl. f. Physiol., 19, N:o 11, 1905.
[10] Bio-chemical Journ., 1, p, 123, 1906.
[11] Journ. of Physiol., 30, p. 155, 1903.

shown, unless the spleen had been previously digested with weak acetic acid. After this pretreatment the enzyme could be obtained from the residue.

New investigations on digestion with alkali and acid.

In the first place I have carried out some new experiments with fresh organs, which fully confirm the observations that the digestion of many organs is much more powerful with acid than with alkali. Equal pieces of the same organ were digested with equal volumes of 0,25 % Na_2CO_3 and of 0,2 % acetic acid. Toluol was used as antiseptic. After a certain time (1—2 days) at 37° equal volumes of tannic acid solutions were added, and the nitrogen was determined in equal volumes of the filtrates. The figures given represent the number of c. c. decinormal acid wanted for neutralising the ammonia obtained.

	Rabbit's liver	Rabbit's kidney	Rabbit's spleen	Calf's thymus	Bull's testicle
Dig. with Na_2CO_3	3,15	3,6	0,6	4,75	22,7
acet. acid	18,2	19,55	4,25	47,6	31,35

The results do not allow of any conclusions as to the relative amount of autolysis of different organs, because the amounts used for the experiments were not the same for different organs.

Further experiments on the digestion with alkali.

Equal pieces were digested as above with alkali and with acid. After 24 hours at 37° the fluids were poured off, and the pieces were washed thoroughly with water. Then *both were digested with equal volumes of* 0,25 % *Na_2CO_3 solution*, and after 48 hours at 37° the amount of digestion was determined as above:

Pretreatment with:	Ox's spleen	Ox's liver	Cat's liver	Rabbit's liver
Na_2CO_3	24,5	5,1	3,9	1,35
acetic acid	63,7	10,2	14	4,55

	Rabbit's kidney	Dog's kidney	Calf's thymus	Bull's testicle	Ox's muscle
Na_2CO_3	0,9	4,3	7,85	7,9	2,35
acetic acid	3,15	8,5	17,8	14,9	3,1

Notwithstanding the fact that during the pretreatment with acetic acid more of the digestible substance was removed than during the pretreatment with alkali, the results show a *much stronger digestion in alkaline media after pretreatment with acid than with alkali.* No comparison between different organs can be allowed, since the amounts taken for the experiments were not the same.

Since it might be suggested that the above results depend upon the solid substance of the organs absorbing more enzyme from an acid than from an alkaline medium (during the pretreatment), or upon the acetic acid producing a change in the proteids so as to render them more readily digestible, I have repeated the above experiment as far as the spleen is concerned under such conditions that no enzyme was removed, and that added casein supplied the main substrate for the digestion instead of the organ itself.

15 gm. of minced spleen were treated for 20 hours at 37^0 in one case with 20 c.c. of 0,25 % Na_2CO_3 solution and in another with 20 c.c. of 0,2 % acetic acid. Then to the former sample 20 c.c. of the acetic acid and to the latter 20 c.c. of the Na_2CO_3 solution were added, whereupon both were digested with 200 c.c. of 2,5 % caseinsolution in 0,25 % Na_2CO_3 for 24 hours. The amount of digestion was determined as above with tannic acid, and 200 c.c. filtrate were taken for each estimation. After deduction of the amount of digestion taken place during the pretreatment the results were:

After pretreatment with	Na_2CO_3	24,8
	acetic acid	81,1

In this case the difference is even more pronounced than before. Therefore I think the conclusion is justified that either

some enzyme acting in an alkaline medium (α-protease) is prevented from acting by pretreatment with alkali, or some enzyme is rendered activ by the pretreatment with acid.

In order to be able to decide whether any change in the amount of enzyme present takes place, I have tried to carry out a rough quantitative estimation of the amount of active enzyme present in the spleensubstance by digesting it together with a very large amount of caseinsolution. In the case of trypsinsolutions this method has been found to give very good results, as I have been able to show in a previous paper[1]. In the case of *solutions* of α-protease the effect shows a tendency to increase more rapidly than the amount of enzyme added (p. 9). If spleensubstance must be used, the question is necessarily more complicated. I have tried the method in a case, where spleensubstance was used, which had been ground with an equal weight of silversand in a Rowland's desintegrator. The caseinsolution used contained 2,5 % in 0,25 % Na CO_3. The digesting samples were made up of

	20 Sandspleenpulp	+ 45 H_2O	+ 200 Cas.	
and	10	+ 50	+ 200	
The former sample gave the effect				13,25
» latter	»	»	»	7,15

In this experiment the effect therefore was very nearly proportional to the amount of enzyme added. For my purpose it is, however, not quite necessary that the effect should be proportional to the amount of enzyme; in order to show an increase or a diminution of the amount of enzyme it is sufficient to know that a larger effect necessarily must correspond to a larger amount of active enzyme than a lower effect.

The following experiments were all carried out with 200 c.c. of caseinsolution. The spleens used were perfused with about 10 liters of salinesolution through the vascular system in order

[1] Journ. of Physiol., 32, p. 474, 1905.

as far as possible to remove the serum. In exp. 1 and 2 10 grams of the minced spleenpulp was used for each sample; in 3, 4, 5, 6 the pulp was ground with an equal weight of silver-sand in a ROWLAND's desintegrator, and 20 grams of the mass were taken. Digestion with casein was carried out in some samples at once (without pretreatment), in others after treatment with 25 c.c. of 0,25 % Na_2CO_3 solution for 24 hours at 37° or with 25 c.c. of 0,2 % of acetic acid for about 18 hours or less (5 hours in exp. 6). In order to obtain the same volume and the same degree of alkalinity in all samples during the digestion with casein 25 c.c. of acid were added to the samples which had been treated with alkali, and 25 c.c. of alkali to those which had been treated with acid. To those which were digested with casein at once 25 c.c. of acid and 25 c.c. of alkali were added. The strength of the alkalisolution was only 0,1 % in exp. 4. In exp. 5 and 6 the pretreatment with acid was carried out as before; then 4 c.c. of 2 % Na_2CO_3 solution were added, and the specimens were treated with alkali in exp. 5 for 24 hours and in exp. 6 for 12 hours.

125 c.c. of 10 % tannic acid solution and 200 c.c. of filtrate were taken for each estimation. The non-precipitable nitrogen in the casein used and that present in the spleensubstance at the beginning of the digestion with casein were deducted from the figures directly obtained. The results were as follows:

	Digestion with casein:		
	At once	After treatment with alkali	After treatment with acid
Exp. 1.	26,05	7,95	40,65
" 2.	39,25	—	52,05
" 3.	25,4	5,75	33,5
" 4.	18,55	6,75	18,95

	Digestion with casein:	
	After treatment with acid	After treatment with acid and with alkali
Exp. 5.	18,95	17,75
" 6.	29,8	27,75

From these results the following conclusions can be drawn:

1. Direct treatment with alkali has a diminishing influence upon the amount of α-protease present in the spleen (exp. 1, 3, 4).

2. Direct treatment with acid as a rule increases the amount of activ enzyme (exp. 1, 2, 3). Only in exp. 4 the result was the same as before the treatment with acid. In no case a diminution was observed.

3. Treatment with alkali after previous treatment with acid does not materially weaken the enzyme (exp. 5, 6).

4. No distinct difference was found between the experiments carried out with minced spleen (1, 2) and those carried out with ground spleensubstance (3, 4).

How is the increasing effect of the treatment with acid upon the α-protease brought about?

In order to try whether the effect of the treatment with acid be in any way due to the β-protease called into action by the acid, I have determined whether the *amount* of β-protease present during the treatment with acetic acid has any influence upon the amount of α-protease rendered activ.

15 grams of minced spleen were treated with a mixture of α- and β-protease in 0,2 % acetic acid during 5 hours at 37°. A control was treated with acetic acid in watery solution. Then to the former sample 25 c.c. H_2O and to the latter 25 c.c. of the enzymesolution were added, and the amount of α-protease was determined with 200 c.c. caseinsolution as above.

The results were:

Pretreatment with acetic acid and β-protease	71
alone	73,4

The β-protease therefore had no increasing influence upon the α-protease. The effect in the sample to which enzymemixture was added is even a little less than in the other — in all probability this is due to the destructive influence of the acetic

acid upon the α-protease during the pretreatment. The experiment was repeated with only $^1/_2$ hour's pretreatment with acid, and no difference was found between the two samples.

Having thus failed to find any influence of the β-protease, I tried whether an addition of α-protease to spleensubstance had a different effect before and after treatment of the spleensubstance with acetic acid. The α-protease, which contained at the same time β-protease, had been obtained by treating minced spleen with 0,1 % acetic acid at 37^0 for about 2 days, filtering, precipitating by saturating with Am_2SO_4 and dialysing away the salt. In order, if possible, to obtain a method for determining the amount of enzyme present, I have tried whether this enzyme like trypsin gives an effect proportional to the amount of enzyme added, when allowed to act upon casein in excess. This was found not strictly to be the case, in as much as the effect shows a tendency to increase more rapidly than the enzyme. In all probability this depends upon the enzyme being destroyed during the digestion more rapidly in a dilute than in a concentrated solution. Only enzyme which has been kept for some time, or which has been obtained by prolonged digestion of the spleensubstance with acetic acid, gives an effect strictly proportional to the amount of enzyme added. In the following experiment the enzyme *a* had been obtained by one day's treatment with acetic acid, and enzyme *b* by acting upon the not dissolved residue of the same spleen with acetic acid for another 4 days. 200 c.c. of caseinsolution were taken for each digestion:

	a.	b.
10 c.c.	26,1	11,35
20 c.c.	60,45	23,8

The spleen which was used for trying the effect of α-protease in the presence of spleensubstance, had been washed through the vascular system with a large amount of saline in order to remove the serum, which I have found to contain an antibody

of the α-protease[1]. Of the minced pulp 5 gm. were digested in one case with, and in another without added protease. As substrate 200 c.c. of caseinsolution was added. The difference between the amount of digestion in the two specimens represents the effect of the protease added. The experiment was repeated after the spleen had been treated with acetic acid for 20 hours at 37^0 and neutralised. The effect of the same amount of α-protease was found to be:

before treatment with acid	23,35
after » » »	31,6.

Therefore *the effect of added α-protease is decidedly larger after treatment of the spleensubstance with acetic acid than before.*

Other experiments on the same subject were carried out with juice pressed from spleen instead of spleenpulp. The spleen used for preparing the juice had been washed through the vascular system with much saline, minced, mixed with silversand, ground, and mixed with kieselguhr. In order to keep the reaction alkaline during the preparation of the juice, a little light magnesiumoxide was added. The digestion took place in the presence of 200 c.c. of caseinsolution as usual. After deduction of the amount of non-precipitable nitrogen present in the samples before the digestion and in the control without juice and protease the results were:

Juice taken for digestion at once:

	Juice + protease	Juice alone	Difference
5 c.c. of juice	37,0	4,05	32,95
10 » »	38,55	6,5	32,05
15 » »	38,8	8,2	30,6

The amount of α-protease added gave by itself the result 37,75, and therefore the differences in the above table representing the effect of the protease in the presence of spleenjuice are all lower than the effect of the protease by itself.

[1] Journ. of Physiol., 30, p. 155, 1903.

Juice treated with alkali for 24 hours:

	Juice + protease	Juice alone	Difference
15 c.c. of juice	25,5	6,45	19,05

Juice treated with acetic acid for 24 hours:

	Juice + protease	Juice alone	Difference
15 c.c. of juice	30,95	9,85	21,1.

The protease used in these two cases gave by itself the figure 21,15, and therefore the juice treated with alkali still produced some diminution in the effect of the protease, whilst the one treated with acid was without influence.

In another experiment the effect of protease added to spleen-juice before and after treatment with acid was:

before	23,5
after	27,35.

All the experiments on the effect of α-protease in the presense of spleenpulp or spleenjuice therefore bear out the fact that the digestion is stronger after treatment of the spleenpulp or the spleenjuice with acetic acid than before. In addition, the first experiment with the juice shows that the juice before treatment with acid checks the action of the protease but not after the treatment. Therefore I think the conclusion is justified that the *fresh spleen contains a substance capable of checking the action of the α-protease, which substance is destroyed by the action of acid.* This substance seems to be of the nature of an antibody, because it shows a somewhat stronger effect when added to the enzyme before the substrate than it does when added after. In one case the neutralising effect of the spleenjuice when added before the casein was 7,15, and, when added after, 6,25. Analogous results I have obtained with the tryptic antibody in the serum[1] and with the antibody of the α-protease in the serum (p. 16).

[1] Journ. of Physiol., 32, p. 390, 1905.

The presence in the spleen of a substance capable of neutralising the α-protease accounts for the results obtained in the above experiments with spleensubstance (p. 8). Since the fresh organ shows both proteolytic activity and antiaction, the enzyme and the antibody must be partly present uncombined, in all probability attached to some other colloidal substances, which prevent their coming into contact with each other. Therefore, if the fresh organ is at once digested with casein, then the casein is able during the digestion to combine with some of the free enzyme and is acted on by it. Again, if the organ is treated with alkali before the digestion with casein, then more of the enzyme must combine with the antibody. *This accounts for the diminution of activity by direct treatment with alkali.* If the spleen is treated with acid before the digestion with casein, then the antibody is destroyed before it can combine with the enzyne. *This is the reason why treatment with acid appears to increase the amount of α-protease.* During the treatment with acetic acid always some of the enzyme is destroyed, and therefore the treatment with acid does not always result in an increase of the proteolysis with alkali. The fact that the antibody is destroyed by acid, accounts for the observation that *after treatment with acid subsequent treatment with alkali does not materially diminish the activity of the enzyme.*

Experiments with spleenjuice kept alkaline during the preparation show that the amount of α-protease in the juice is considerably less than the amount obtained from an equal weight of spleanpulp. Thus the proteolysis obtained with 15 c. c. of fresh juice was in two cases 8.2 and 7.55, whilst the figures obtained with 10 grams of fresh pulp were 26.05—39.25—25.4—18.55 (p. 7). This, in all probability, depends upon the enzyme and the α-protease combining more readily in the liquid juice than in the semisolid cells. In consequence, the fall in activity under direct treatment with alkali and the rise under treatment with acid are much less marked in the juice than in

the pulp. In one experiment with juice the effect of the α-protease in 15 c. c. of juice diminished during 24 hours with alkali from 8.2 to 6.45 and increased during 24 hours with acid from 8.2 to 9.85. The corresponding figures obtained with the pulp are given p. 7. For the same reason the diminution in the amount of active α-protease in the pulp must be less during the second day of treatment with alkali than during the first. In one case the effect of 15 gm. of pulp diminished during the second day from 24.8 to 22.7, which is a very small difference as compared with the fall during the first day (p. 7).

Antibody of the α-protease present in the serum.

The antibody found in the spleen washed free from serum in all probability is the same as the one I have previously found in the normal serum, and which is found together with the serumalbumin, when the serum is fractionated with Am_2SO_4 [1]. This antibody is not a very strong one; it is not nearly so effective as the tryptic antibody present in the serum [2]. In addition, it is readily destroyed by 0.2 % acetic acid, as shown by the following experiments:

The serumalbumin, dialysed free from salt, was kept with 0.2 % acetic acid at 37° as recorded. Then the acetic acid was dialysed away. The spleenenzyme obtained as above (p. 9) and the antibody or an equal volume of water were kept for about one hour at 37° before the substrate (200 c. c. casein-solution) was added.

Without antibody	39.75
With 5 c. c. intact antibody	19.5
» antibody kept at 37° for 24 hours without acid	25.6
with	39.8.

[1] Journ. of Physiol., 30, p. 155, 1903.
[2] Ibid., 32, p. 399, 1905.

Therefore the antibody is completely destroyed by 0.2 % acetic acid at 37° for 24 hours. Even heating to 37° for 24 hours without acid has a somewhat injurious effect.

For the destruction of the antibody in one case treatment with 0.2 % acid at 37° for 3 hours was found to be sufficient:

Without antibody 48.35

With 5 c. c. antib. heated for 3 hours with acid . . 48.5

Since the antibody is destroyed with acid and antibodies according to the view generally accepted act by combining with the substance they neutralise, it might be suggested that the antibody in the compound thus formed could be destroyed with acetic acid leaving the protease free. In that case a proteasesolution after treatment with antibody in a neutral fluid ought to increase in digestive power by treatment with acetic acid. All experiments which I have carried out with a view of settling this question have failed to give a positive result. In all cases I have found a steady fall of the power of the enzyme under the influence of acetic acid, and the fall seems to be proportional to the fall taking place in the absence of any antibody, as shown by the following experiment:

200 c. c. caseinsolution in 0.2 % Na_2CO_3 were used as substrate, and the same amount of acetic acid was added to all samples, although the acid in the samples, which were digested at once, was immediately neutralised by the alkali in the caseinsolution.

	at once	after 16 hours with acid
With 5 c. c. destroyed antibody .	43.0	25.2
intact antibody . .	23.8	14.4

Destroyed antibody was used instead of no antibody in order to make the amount of proteids the same in all samples.

From this experiment and similar ones, which have not been recorded, I must draw the inference that *by the action of acetic acid upon the compound α-protease-antibody no enzyme can be liberated.* Quite the same result has been obtained with the

tryptic antibody, which likewise is destroyed by acetic acid, although not so readily as the antibody of the α-protease.

The antibody of the α-protease neutralises this enzyme, when allowed to act upon it in neutral (or alkaline) solution, but owing to its destruction it is not nearly so effective, when made to act upon this enzyme in acid solution. Upon the β-protease the serumalbumin is practically without effect. The slight diminution of the digestion with acid, which has been obtained after pretreatment at neutral reaction, might depend upon the α-protease being able to exercice a weak action in acid solution too. The action of serumalbumin upon the two enzymes is illustrated by the following experiments:

Serumalbumin was allowed to act upon a mixture of both enzymes in neutral and in acid solution (0.2 % acetic acid) for 3 hours 37°. Controlspecimens were kept with the same volume of water. Then the digestive power of the fluids was determined with 200 c. c. of caseinsolution at alkaline reaction (α-protease) and at an acid reaction (5 c. c. of 20 % acet. acid to 200 c. c. of caseinsol.). The amount of digestion with acid is mainly due to the β-protease.

Exp. 1.

	Pretreatment at neutral reaction	Pretreatment at acid reaction
Digestion with alkali:		
Without antibody	12,45	13,0
With »	4,7	10,8
Digestion with acid:		
Without antibody	7,55	7,2
With »	6,35	7,55

Exp. 2.

Digestion with alkali:		
Without antibody	12,6	—
With »	4,5	—
Digestion with acid:		
Without antibody	17,35	—
With »	15	

In another experiment the protease and the antibody were kept with acid for 24 hours before the casein was added:

Digestion with alkali:

Without antibody	39,75
With	40,2

In the case of the tryptic antibody present in the serum I have found that the neutralising effect of the antibody increases for some time, if it is allowed to act upon the enzyme before the substrate is added. Therefore the effect of the antibody is least marked, when the substrate is mixed with the enzyme before the antibody is added[1]. Experiments with the α-protease and its antibody in the serum likewise show that the antibody neutralises more enzyme when it is added to the enzyme before the enzyme than after:

Without antibody	24,45
Casein added before the antibody	17,4
Antibody added before the casein	13,25

This observation gives an explanation of the fact that spleen-pulp, when digested with casein, gives a larger proteolytic effect, when the digestion is carried out with the fresh spleen than after treatment with alkali (p. 8 and 12).

A case entirely analogous to the above experiments on the influence of spleensubstance upon the proteolytic effect of added α-protease would be obtained by trying the effect of α-protease in the presence of a mixture of α-protease and serum-albumin before and after the mixture has been acted upon by acetic acid.

In the following experiment the effect of added protease was tried without any treatment with acid. The mixture was made up of 15 c. c. antib. + 25 c. c. prot., and the amount of protease added was 25 c. c.:

Mixture without added prot. . . .	7,25
Mixture + protease	13,8
Protease without mixture	10,6.

[1] Journ. of Physiol., 32, p. 390, 1905.

Therefore the effect of the protease by itself was 10,6, and in the presence of the mixture, 6.55.

In another experiment the effect was estimated before and after the mixture had been treated with acid.

	before	after
Mixture without added protease . .	3,65	3,6
Mixture + protease	10,75	14,3
Protease without mixture	10,45	10,45.

Therefore the effect of the protease in the presence of the mixture was 7,1 before and 10,7 after the mixture had been treated with acid, and the result corresponds entirely to the results obtained with spleensubstance and spleenjuice (p. 9—11.).

By the above investigations I think it is shown that the spleen in all probability contains a substance which checks the action of the α-protease, and which is destroyed by acetic acid. Since normal serum contains a substance in all probability identical with the one observed in the spleen, it might be suggested that the checking influence of the spleen could be due to serum left behind in the organ. It is very difficult to prove that this is not the case, and the reason that makes me believe that the antibody is attached to the organ itself, is that in all cases, where the checking substance was observed, the spleen had been previously perfused with very large amounts of saline (about 10 liters), by which the serum ought to have been washed away. The subject requires further investigation.

The presence of an antibody that can be destroyed by acetic acid in other organs might be the explanation of the fact that several organs give a stronger digestion with alkali after pretreatment with acid than with alkali, as shown p. 4. Of the organs used for these experiments those of the rabbit (liver and kidneys) had been taken from an animal which had been washed free from blood through the vascular system.

The antiaction of the serum does not seem to be specific, the normal serum of the pig and horse checking the proteolytic activity of α-protease, obtained from the ox spleen[1].

[1] Journ. of Physiol., 30, p. 168, 1903.

Are the α- and β-protease of the spleen of the ox different enzymes?

The products of digestion of the β-protease as found by LEATHES[1] and those of the α-protease as investigated by CATHCART[2] are roughly the same. It would, however, be rash from this fact to conclude that the two enzymes be identical. One might as well suggest that hydrochloric and sulphuric acid be identical, because they split up proteids in the same way. If the two enzymes were identical, then one must expect that the ratio between the proteolytic effect with alkali and with acid should always be the same, after the antibody present has been destroyed. Therefore one should always expect either stronger effect with alkali or with acid. This has not been found to be the case, as illustrated by the following observations:

1. In the ground spleenpulp the proteolytic effect after treatment with acid has been found as a rule to be somewhat stronger with acid than with alkali:

	1.	2.	3.	4.
With alkali	40,65	52,05	33,5	18,95
acid	45,6	49,8	39,85	32,8.

2. The solution obtained by treating minced spleen with acetic acid and precipitating the filtrate with Am_2SO_4 digests more strongly sometimes with alkali sometimes with acid:

	1.	2.
With alkali	12,45	12,6
» acid	7,55	17,35.

Even the same spleen may on different treatment give different results. Equal weights of the same spleen were acted upon with acetic acid for 20 hours, whereupon one sample (a) was made slightly alkaline, whilst the other (b) remained acid, and both were kept at 37^0 for another 2 days. Then the enzymemixture was prepared as usual:

[1] Ibid., 28, p. 360, 1902.
[2] Ibid., 32, p. 299, 1905.

	a.	b.
With alkali	9,3	6,75
» acid	7,3	9,2.

3. After treatment of the spleen with acetic acid one can with NaCl-solution extract from the residue a mixture of enzymes, which acts more strongly sometimes with alkali sometimes with acid. (Journ. of Physiol. 30, p. 159).

4. Spleenjuice obtained by grinding and pressing, as done by BUCHNER, gives after treatment with acid a far stronger digestion with acid than with alkali.

	1.	2.
With alkali	9,85	5,65
» acid	42,45	40,75.

This is in full agreement with my previous results (Journ. of Physiol. 30, p. 171). From spleenjuice I have by precipitating with acetic acid and digesting the precipitate with acetic acid obtained a solution which gave no digestion with alkali.

	1.	2.
With alkali	0,0	0,6
» acid	15,2	15,6.

Another remarkable difference between the two enzymes is their different behaviour to serumalbumin (obtained with Am_2SO_4), in as much as the serumalbumin, when made to act upon the enzymes in a neutral fluid, exercises a marked antiaction upon the α-protease, whilst the β-protease is hardly influenced at all.

I do not see my way to explain these observations except by assuming that the spleen contains at least two different proteolytic enzymes, the one acting principally in an alkaline medium, the other in an acid. According to the above experiments a preparation can be obtained, which acts only with acid (β-protease free from α-protease), whilst no preparation has been obtained, which acted only with alkali.

With regard to the β-protease it is remarkable how easely it is rendered inactive by direct treatment of the spleensubstance with alkali. I give figures obtained by digestion af 10 gm. of

spleenpulp with casein in the presence of acetic acid at once and after 24 hours with 0,25 % Na_2CO_3.

	1.	2.	3.
At once	84.6	80,8	76,55
After treatment with alkali	10,2	13,6	14,85.

This might be the effect of the destructive influence of the alkali, since a remarkable destruction by alkali has been observed even on the enzyme obtained after treatment with acid. The subject requires further investigation.

The influence of the reaction on the proteolysis of the spleen can be summarised as follows:

1. Direct proteolysis with alkali is considerably weaker than direct proteolysis with acid.

2. After direct treatment with alkali the proteolysis is very much diminished both with alkali and with acid.

3. After direct treatment with acid the proteolysis with alkali is markedly improved and the proteolysis is found to be about the same with alkali and with acid.

4. This effect of the treatment with acid depends upon the acid destroying an antibody, which would have checked the proteolysis with alkali, had it not been destroyed.

Experiments with liver, kidneys, thymus, testicles, muscles show that previous treatment with acid improves the autolysis with alkali. Further experiments will decide whether the explanation of this fact is the same as in the case of the spleen.

From the above results it appears that the reaction most favourable for the autolysis of the organs tried is either acid reaction throughout the whole process or acid reaction during some 20 hours and then alkaline.

AN ANTITRYPTIC EFFECT OF CHARCOAL AND A COMPARISON BETWEEN THE ACTION OF CHARCOAL AND THAT OF THE TRYPTIC ANTIBODY IN THE SERUM.

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By chance I recently observed that charcoal, when added to a digesting fluid of trypsin and casein, produces a very marked diminution of the tryptic effect. There can hardly be any doubt that this depends upon the trypsin being taken up by the charcoal, thus being prevented from acting upon casein. It is a well known fact that charcoal readily condenses gases on its surface, and likewise takes up colouring matters from solutions. That other solid substances and especially hydrogels, have got the power of taking up crystalloids from their solutions has been pointed out by several investigators (v. Bemmelen,[1] Schmidt,[2] Biltz,[3] Bayliss,[4] and others). This process has been termed adsorption (Du Bois Reymond, Ostwald).

As to the laws of adsorption very little is known. Henry's rule, which has been found to be valid for the distribution of a soluble substance between two fluids, which implies that the ratio between the concentration in the two solvents is a constant ($\frac{C_1}{C_2} = k$), when equilibrium has been reached, does not apply to the adsorption. In the case of adsorption, a state of equilibrium seems to be arrived at sooner or later; if C_{ads} is the concentration in the solid substance and C_{fl} the concentration in the fluid, then $\frac{C_{ads}}{C_{fl}}$ is not a constant, but diminishes as the total amount of the originally dissolved substance is increased. This means that the solid takes up relatively more from

1. *Zeitschr. anorg. Chem.*, Vol. XXIII, p. 321, 1900.
2. *Zeitschr. physik. Chem.*, Vol. XV, p. 60, 1894.
3. *Chemiker Zeitung*, Vol. XXIX, No. 25, p. 325, 1905.
4. *Bio-Chem. Journ.*, Vol. I, p. 175, 1906.

a dilute than from a concentrated solution. The way in which the ratio $\frac{C_{ads}}{C_{fl}}$ varies with the total amount of dissolved substance is different in different cases, and no general rule has been found. As to other factors which influence the adsorption v. Bemmelen points out the following :—(1) the adsorbing substance, (2) the solvent, (3) the substance to be adsorbed, (4) the state of its molecules, (5) the temperature, without being able to give any general rules as to their influence.

In the cases where the adsorption of colloids has been investigated, the influence of the amount of colloid upon the ratio $\frac{C_{ads}}{C_{fl}}$ has been found to be roughly the same as for crystalloids (Bayliss). In addition, v. Bemmelen observed that the adsorption of colloids (*e.g.*, As_2S_3) may sometimes be complete. Bayliss found that the total amount of congo-red taken up by paper is less the higher the temperature, and he also observed that the congo-red can be completely removed from the paper by repeated extraction with water.

Adsorption of enzymes by solids, and particularly by charcoal, has been observed by Glaessner,[1] Dauwe,[2] and others, but no investigations as to the conditions and quantitative relations of the adsorption have so far been published.

My experiments on the adsorption of trypsin by animal charcoal were carried out with a view to make out whether there exists any analogy between the action of the charcoal upon trypsin and the action of the tryptic-antibody in the normal serum. The animal charcoal used had been prepared from bones, and the phosphates had been removed by repeated heating with HCl. The trypsin had been obtained as given in a previous paper[3]. The digestive power of the trypsin was estimated as described in the same paper, and the figures given represent the number of cubic centimetres of decinormal acid required to neutralise the ammonia obtained from equal volumes of the tannic acid filtrates.

1. *Hofmeisters Beiträge*, Vol. I, p. 1, 1902.
2. *Ibid.*, Vol. VI, p. 427, 1906.
3. Hedin, 'Observations on the action of trypsin,' *Journ. of Physiol.*, Vol. XXXII, p 486, 1905.

Adsorption Without the Presence of Proteid

In the first place it was found that the presence of a proteid (*e.g.* casein) materially influences the process of adsorption, and therefore some experiments were carried out in which only the small amount of proteid contained in the trypsin solution was present during the process of adsorption. Thereupon the charcoal was filtered off, and the digestive power of the filtrate was tested with casein.

Experiment I.—Two mixtures containing, the one 2 gr. charcoal + 80 c.c. tryps. (A), and the other 0·1 gr. charcoal + 80 c.c. tryps. (B), were kept at 20° as recorded below. Thereupon, equal volumes of the filtered samples were made to act upon 50 c.c. of 2·5% casein solution at 37° for 24 hours. 25 c.c. of 10% tannic acid solution were added, and 50 c.c. filtrate were taken for each estimation. The trypsin used for each estimation would, without previous treatment with charcoal and with a sufficient amount of casein, have given the result 240.

	A.	B.
1 hour	0	55·4
4 hours	0	53·75
24 „ ...	0 ...	46·75
48 „ ...	0 ...	43·5

The influence of the amount of charcoal is evident, the adsorption in A with 2 gr. of charcoal being complete after one hour, and in B with 0·1 gr. equilibrium being reached in 24 hours. The small drop observed after the expiration of this time may depend upon destruction of enzyme, as shown by the fact that samples of the same enzyme having been kept without charcoal for two days at 0°, gave 38·6, and at 20° 34·2.

The influence of the temperature was examined by putting the mixture B (after 48 hours at 20°), at 37° for 24 hours. The result was :

After 48 hours at 20°	43·5
After 48 hours at 20° and 24 hours at 37°	2·3

Therefore, if the temperature is raised after equilibrium has been reached at a low temperature, then more trypsin is adsorbed.

Experiment II shows the influence of the temperature and of the time, low temperatures being used in order, as far as possible, to prevent destruction of the trypsin. Samples of the same mixture were kept at 0° and at 20°, as given below. Then the filtrates were tested as above. Without charcoal 120.

	0°	20°
24 hours ...	22·3 ...	4·5
48 „ ...	7·95 ...	0·2

The experiment shows that more trypsin is adsorbed at the higher temperature. The equilibrium was not reached in 24 hours.

Experiment III was carried out at 20°, and only the amount of charcoal was varied.

0·5 gr. charcoal ...	0	0·05 gr. charcoal ...	25·75
0·1 gr. „ ...	3·75	Without „ ...	750

This experiment verifies the rule that $\frac{C_{ads}}{C_{fl}}$ diminishes as the total amount of enzyme is increased, or (which comes to the same) as the amount of charcoal is diminished. A consequence of this rule is, that when the amount of trypsin is the same and the amount of charcoal is diminished, then the charcoal approaches by degrees a state of saturation, where no more trypsin is adsorbed. I have been able to verify this by keeping mixtures of charcoal and trypsin at 20° for 24 hours, whereupon fresh trypsin was added to equal volumes of the fluids before (A) and after (B) filtration. If the charcoal is not saturated, then A gives less digestion than B, and at saturation both give the same result.

0·025 gr. charcoal	A.	28
	B.	36·3
0.0125 gr. „	A.	49·8
	B.	52·65

In this experiment, 100 c.c. of casein solution was added as substrate. The result shows that saturation was nearly reached in the mixture with 0·0125 gr. charcoal.

The above results are remarkable as showing how readily charcoal effects a complete adsorption of trypsin. Similar observations have

been made before by v. Bemmelen (page 485), but to my knowledge complete adsorption has not been observed for crystalloids.

According to the above experiments, more trypsin is adsorbed the higher the temperature. In the case of the adsorption of congo-red by paper, the influence of the temperature is the reverse (Bayliss, p. 485).

Influence of the Presence of the Charcoal During the Digestion

In all the above experiments, the charcoal used for the adsorption was removed before the casein was added. The result of the digestion becomes somewhat different if the charcoal is not removed, as proved by the following experiment.

Experiment IV.—The trypsin and the charcoal were allowed to act upon each other as recorded, then equal volumes of the fluids were digested with 50 c.c. of casein solution in set A, after filtering off the charcoal, and in set B, in the presence of the same.

				A.	B.
0·5 gr. charcoal	24 hours at	37°	...	0	11·7
0·25 ,,	,,	37°	...	0	18·45
0·5 ,,	,,	17°	...	0	44·1
0·5 ,,	48 hours at	37°	...	0	5·6
0·25 ,,	,,	37°	...	0	8·5

The amount of trypsin rendered inactive by the charcoal was very considerable, since the trypsin used for each digestion, without charcoal and with an excess of casein, would have given the result 960. No sample of set A showed any tryptic effect, which implies, that before the addition of the casein the adsorption was complete in all samples. The digestion taken place in the samples of Set B, therefore, must depend upon the casein being able to deprive the charcoal of a small amount of the trypsin adsorbed. This is confirmed by the following experiment.

Experiment V.—An amount of trypsin, which corresponded to the effect 2400, was kept with 0·5 gr. charcoal at 37° for 24 hours.

Then the adsorption was complete, 5 c.c. of filtrate, in 24 hours,

with 50 c.c. of casein solution, giving the effect 0·7. The charcoal was thoroughly washed and digested with 50 c.c. of casein solution. The effect was 30·6.

That the trypsin is attached to the casein before the digestion, is proved by the following experiment.

Experiment VI.—A mixture of 0·5 gr. charcoal + 80 c.c. tryps., was kept at 20° for five hours. The adsorption was then complete. To 50 c.c. of the mixture 300 c.c. of casein solution at 37° were added, and the whole was kept at 37° for one hour. Then part of the mixture was repeatedly filtered in order to remove the charcoal. 50 c.c. of the casein-charcoal-trypsin mixture and 50 c.c. of the filtrate were digested with the following result :—

With charcoal	25·1
Without „	23·2

Another experiment with other proportions gave

With charcoal	65·8
Without „	64·4

The experiments show that all the trypsin available had been transferred from the charcoal to the casein during one hour at 37°.

Therefore, the casein undoubtedly is capable of taking back some of the trypsin already adsorbed by the charcoal. The amount taken back in the above experiments was 1-15% of the amount originally adsorbed. The amount of trypsin rendered active by addition of casein after complete adsorption by charcoal depends upon

1.—The amount of charcoal used, less trypsin being rendered active the more charcoal present.

2.—The temperature at which the adsorption has taken place, less trypsin being rendered active the higher the temperature.

3.—The time of interaction between the trypsin and the charcoal, less trypsin being rendered active the longer the time.

This is all borne out by Experiment IV.

The influence of the temperature was tried in another experiment, where lower temperatures were used, in order, as far as possible, to prevent destruction of enzyme.

Experiment VII.—Two equal mixtures were kept for 48 hours, the one at 0°, the other at 20°, whereupon the digesting power was tested after filtering off the charcoal (A) and in the presence of the same (B). The amount of trypsin used, would, without treatment, with charcoal, have given the effect 93.

	A	B
48 hours at 0° ...	0 ...	29·8
48 ,, 20° ...	0 ...	9·8

According to the above investigations, the process, by which trypsin is rendered inactive by charcoal, can be divided up into two different stages: (1) the *taking-up*, or adsorption of the trypsin by the charcoal, and (2) the *fixation* of the same. The amount of trypsin taken up by the charcoal is represented by the difference in digesting power between the trypsin not treated with charcoal, and the filtrate after treatment with charcoal. The process of fixation can most conveniently be studied in experiments with rather a large amount of charcoal, in which case all the trypsin very soon is taken up, whilst the fixation is still in progress. In such cases the amount of trypsin taken up and not fixed is obtained by direct estimation of the digestive power of the mixture.

If very little charcoal is used, then both processes come to a standstill before all trypsin is rendered inactive. This shows that the trypsin is not merely destroyed, because in that case the process would not finish till all trypsin were destroyed. It is very difficult to form a clear view of what is taking place. At present I am inclined to think that the trypsin is attached to the surface of the charcoal by molecular attraction, and subsequently proceeds further into the charcoal by diffusion, thus, by degrees, becoming inaccessible for the proteid. As long as the trypsin is attached to the surface of the charcoal, or merely adsorbed, it can be transferred to added casein, but not when it has entered deeper into the same. This view would account for the fixation going faster the more charcoal is present, *i.e.*, the larger the surface through which the diffusion takes place. It would not be difficult to conceive that a process of diffusion would depend upon the temperature and the time, as found above, for the

process of fixation. In this way it would be easy to understand that only part of the trypsin taken up by the charcoal can be rendered active by addition of casein.

As pointed out, charcoal, if present in sufficient amount, adsorbs trypsin completely, leaving no trypsin in the filtrate when filtered off. On the other hand, if casein is added to the mixture of charcoal and trypsin before filtration, then a considerable amount of the trypsin already adsorbed appears in the filtrate together with the casein. This fact affords a very strong support for the view that trypsin is attached to proteids previous to its action, and it does not seem unlikely that this is a process of adsorption. This would be quite consistent with the fact that proteids do not form any real solutions with water, but suspensions of larger or smaller particles. If the taking-up of trypsin by proteids is a process of adsorption, then it might be expected that the proteids, if present in sufficient amount and in absence of other adsorbing substances, could, like charcoal, completely adsorb the trypsin. In a previous paper I have pointed out that complete combination of the trypsin is required for the explanation of the following two observations :—

1.—The effect of trypsin upon certain proteids is independent of the dilution, and

2.—The effect is the same after the same number of trypsin time units.[1]

Comparison of the Neutralising Power of Charcoal with that of the Tryptic Antibody in the Serum

From the above experiments it is evident that charcoal exercises a very marked checking influence upon the action of trypsin, which influence is very much like the effect produced by the tryptic antibody contained in normal serum. In the case of charcoal, the process of neutralisation can easily be distinguished from the process of digestion, by filtering off the charcoal before the proteid to be digested is added. In the case of the antibody, the two processes cannot be strictly

1. *Journ. of Physiol.*, Vol. XXXII, p. 480, 1905.

distinguished, because the neutralising substance cannot be removed. In order to compare the effect upon trypsin of charcoal and of antibody, one, therefore, has to carry out the digestion in the presence of the neutralising substance in both cases. Investigations of that kind I have previously carried out as far as the antibody is concerned.[1] Corresponding observations with charcoal can be obtained from Experiments IV and VII. Some additional observations are given below. Investigations have been carried out with reference to :—

1.—The time of interaction of trypsin and the neutralising substance.

2.—The temperature.

3.—The dilution.

4.—The saturation of the neutralising substance with trypsin, and

5.—The complete neutralisation of trypsin.

From the above experiments it follows that the *amount of trypsin neutralised is larger, the longer the time and the higher the temperature.* Complementary conclusions can be drawn from the following experiments.

Experiment VIII.—Mixtures of 1 gr. charcoal + 20 c.c. tryps. were kept as given below. Then the digesting power of the mixtures was determined with 50 c.c. of casein solution at 37° for 24 hours.

		17°		37°
3 hours without charcoal	...	43·65	...	43·4
5 min. with charcoal	...	30·5	...	22·9
30 min. ,,	...	25·5	...	lost.
1 hour ,,	...	21·4	...	9·2
3 hours ,,	...	16	...	2·95
3½ hours ,,	...	14·35	...	2·15

Equilibrium was not reached in three hours.

With another enzyme, the following results were obtained.

				20°		37°
18 hours	...	...	...	40·6	...	11·85
19 ,,	...	...	...	39·9	...	9·8

1. See page 474.

The amount of enzyme neutralised was very considerable, since the trypsin used, with an excess of casein, would have given the figure 470. One sample, which had been kept at 37° for 18 hours (corresponding to 11·85), was kept at 20° for 24 hours, and gave then the result 9·6. Another sample, which had been kept at 20° for 18 hours (corresponding to 40·6), lost in digesting power during eight hours at 37° (result = 25·1).

This experiment, and others which have not been recorded, show that a very long time is wanted for reaching the state of equilibrium, where no change takes place. This makes it very difficult to decide whether a diminution in activity be due to adsorption or to destruction of enzyme. Considering the enormous amount of enzyme rendered inactive by the charcoal the fall after 24 hours is however rather insignificant, and I think one is justified in saying that equilibrium is arrived at, although very slowly.

Experiment IX.—In order to find out whether the *dilution* has any influence upon the amount of trypsin rendered inactive by charcoal, specimens were made up as follows :—

0·05 gr. charcoal	+ 20 c.c. trypsin	...	...	... A
0·05 ,,	,,	+ 20 c.c. H_2O	...	B
0·05 ,,	,,	+ 40 c.c. H_2O	...	C.

After 24 hours at 20°, 40 c.c. of water were added to A, and 20 c.c. to B, whereupon the digestive power of the specimens was estimated as usual. The result was :—

A = 78·4 B = 80·3 C = 80·5

and consequently *the dilution had no influence.* Control specimens, which were filtered before the casein was added, showed that the adsorption was not complete.

Experiment X.—Whether charcoal can be *saturated* with trypsin was tried as follows. 0·01 gr. charcoal + 50 c.c. tryps. was kept at 20° for 24 hours, and then at 37° for 24 hours. The digestive power was determined with an excess of casein (200 c.c.), in which case the results obtained are proportional to the amount of active trypsin present.[1]

1. *Journ. of Physiol.*, Vol. XXXII, p. 471-474, 1905.

Three estimations were carried out, namely, of 5 c.c. of the above charcoal-trypsin mixture, of 5 c.c. of this mixture in the presence of 1 c.c. of trypsin, not previously acted upon by charcoal, and of this amount of trypsin alone. The sample, with mixture and trypsin, was kept at 37° for one hour before the casein was added. The results were :—

5 c.c. of mixture	12·1
5 c.c. ,, + 1 c.c. of tryps.	35·3
1 c.c. of trypsin	23·4

Therefore, the effect of the trypsin, by itself, (23·4) was the same as the effect of the trypsin in the presence of the charcoal-trypsin mixture (35·3 — 12·1 = 23·2), which proves that the charcoal in the mixture had not affected the trypsin, and that the *charcoal was saturated with trypsin* before the fresh trypsin was added.

As to the question, *whether trypsin can be completely neutralised* by charcoal, it is sufficient to refer to the above investigations, according to which it is very easy completely to remove all trypsin from a solution, by using a large amount of charcoal and filtering it off before the casein is added, but that *always some tryptic effect has been observed, when the digestion has been carried out in the presence of the charcoal which had absorbed the trypsin.*

The above results, obtained with trypsin and charcoal, as compared with corresponding results obtained with trypsin and its antibody in the serum,[1] show that in all respects tried, there exists a very close agreement between charcoal and the antibody, with regard to their effect upon trypsin. In consequence of this agreement, I am very much inclined to believe that the neutralisation of the trypsin is brought about in the same way in both cases. As I have pointed out above, the neutralisation of trypsin by charcoal implies, not only the *adsorption* or the taking-up of trypsin by the charcoal, but also the *fixation*, or the process by which the trypsin is rendered inaccessible for proteids. In all my experiments with charcoal some trypsin has remained merely adsorbed, and therefore transferable to added casein

1. See page 483.

and active. Accordingly, it is only reasonable to think that the same might have been the case in experiments with the antibody, and, in my opinion, this is a very acceptable explanation of the fact, that it has been found impossible completely to neutralise trypsin by aid of the antibody.

Conclusions

1.—The process of neutralisation, exercised by charcoal upon trypsin, can be divided in two consecutive stages :—

(*a*) The taking-up, or *adsorption* of the trypsin by the charcoal. A complete adsorption of the trypsin can easily be effected by using a sufficient amount of charcoal. The trypsin merely adsorbed is readily transferred to added casein.

(*b*) The *fixation*, or the process by which the trypsin is rendered inaccessible for added casein. The amount of trypsin fixed is larger, the larger the amount of charcoal, the longer the time of interaction and the higher the temperature.

2.—The action of charcoal has been found to agree with that of the tryptic antibody in all respects tried, and therefore the neutralising effect, in all probability, is brought about in the same way in both cases.

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FURTHER OBSERVATIONS ON THE TIME-RELATIONS IN THE ACTION OF TRYPSIN. By S. G. HEDIN.

(*From the Lister Institute of Preventive Medicine, London.*)

Some time ago I published a paper on the action of trypsin, in which it was shown, that if in different digesting specimens everything except the amount of enzyme and the time of digestion was the same, then the effect was the same in all samples for which the amount of enzyme multiplied with the time of digestion was the same[1]. This rule evidently involves that the effect is the same after the expiration of the same number of trypsin time units. The result was obtained by estimating the amount of nitrogen not precipitable by tannic acid in the digested fluid, and the rule was found to hold good for casein, serum albumin, white of egg and even for Witte's peptone, when these substances were digested in solutions which were as neutral as possible.

Casein contains another constituent, which can easily be determined and by which the validity of the above law can also be tried, namely phosphorus. I have estimated the amount of phosphorus which is transformed by the trypsin into such a form that it is no longer precipitable by tannic acid, and I have been able in this way to verify the above time law.

The trypsin was prepared as indicated in my previous paper; the casein solution contained 2·5 % of casein in 0·1 % Na_2CO_3 solution. For the estimation of phosphorus Neumann's method was used[2], and the "effect" represents the number of c.c. of decinormal alkali wanted for the neutralisation, after making the necessary deductions determined in special controls without trypsin and also in some of the trypsin itself.

One c.c. of decinormal alkali corresponds to 0·254 milligrams of P_2O_5. The non-precipitable phosphorus in the trypsin solution was very low. It corresponded to 3·5 c.c. of decinormal alkali in 100 c.c. of trypsin solution. During the digestion each specimen contained 100 c.c. cas. sol. + 30 c.c. (tryps. + H_2O). At the end of the digestion 50 c.c. of

[1] This *Journal*, xxxii. p. 468. 1905.

[2] *Zeitschr. physiol. Chem.* xxxvii. p. 129. 1902.

tannic acid solution were added and 125 c.c. of filtrate were taken for each analysis.

	C.c. of trypsin solution	Time of dig. in hours	Effect
Exp. 1	10	5	26·1
	20	$2\frac{1}{2}$	25·6
	10	$7\frac{1}{2}$	30·45
	30	$2\frac{1}{2}$	31·25
Exp. 2.	10	4	19·1
	20	2	18·35
	5	12	23·35
	10	6	23·6
	20	3	23
	30	2	22·5

One specimen with 10 c.c. of trypsin was digested for 3 days and gave the effect 76, showing that in the above experiments only part of the phosphorus had been split off.

The phosphorus of casein is very readily split off by the action of alkali as shown by Bayliss and Plimmer[1]. According to these authors practically all the phosphorus is rendered soluble in tannic acid by 1 % NaOH at 37° for 24 hours. I have tried whether the alkali used for dissolving the casein has any influence upon the splitting off of phosphorus by digesting at 37° 100 c.c. of 2·5 % casein solution in 0·2 % Na_2CO_3. The phosphoric acid obtained from 125 c.c. of the tannic acid filtrate after 48 hours' digestion corresponded to 5·2 c.c. of decinormal alkali, which figure is considerable as compared with the amount of phosphorus set free by the trypsin in my experiments, and one might not be justified in assuming that the influence of the alkali would be the same with and without the presence of trypsin (cf. Bayliss and Plimmer, p. 459). In order as far as possible to diminish the influence of the alkali I have in my above experiments only used 0·1 % Na_2CO_3 for dissolving the casein and at the same time the time of digestion was confined to some hours.

Summary.

The rule, that the tryptic effect is the same after the expiration of the same number of trypsin time units, which has been previously found to be valid as far as the splitting-off of nitrogen is concerned, is confirmed with regard to the splitting off of phosphorus from the casein molecule.

[1] This *Journal*, xxxii. p. 453. 1906.

ON EXTRACTION BY CASEIN OF TRYPSIN, ADSORBED BY CHARCOAL

By S. G. HEDIN.

From the Lister Institute of Preventive Medicine

(*Received December 3rd*, 1906)

According to recently published investigations of mine, charcoal exercises an anti-tryptic action, which can be divided up into two consecutive stages, namely, (1) the taking up or adsorption of the trypsin by the charcoal, and (2) the fixation of the trypsin.[1] The mere adsorption could, in absence of proteins, very readily be made complete, as shown by the fact that, after filtering off the charcoal, the filtrate showed no tryptic activity. On the other hand the fixation was never found to be complete as borne out by the observation that a mixture of charcoal and trypsin, even after complete adsorption, gave some tryptic effect with added casein. This was found to depend upon the casein extracting part of the enzyme from the charcoal. The tryptic effect obtained after complete adsorption therefore represents in a way the amount of trypsin adsorbed but not fixed. Furthermore it could be shown that, after proper treatment with casein solution and filtering off the charcoal, the filtrate gave the same tryptic effect as the non-filtered charcoal-trypsin-casein mixture. This proves that all the trypsin that is able to act upon the casein can be made to pass through the filter together with the casein—pointing to some kind of combination being formed between casein and the trypsin. This is in full agreement with the view generally accepted, according to which the action of enzymes is made up of two consecutive stages—(1) combination of the enzyme with the substrate, and (2) breaking up of the latter.

1. *Bio-Chemical Journal*, I, p. 484, 1906.

Up to the present time it has not been possible, experimentally, to distinguish between these two stages, or to study the one independently of the other. The experiments referred to open, I think, a way to studying the process of combination by itself.

If a mixture of a sufficient amount of charcoal and trypsin (approximately free from proteins) is kept for twenty-four to forty-eight hours, then the adsorption will be completed, and the fixation of the trypsin to the charcoal will be so nearly finished that the amount of trypsin adsorbed but not fixed can be considered as constant, anyhow during a short interval of time (one to two hours). By means of added casein-solution, part of the trypsin can be extracted from the charcoal and attached to the casein, and this process can be interrupted at any time by filtering off the charcoal. The tryptic effect obtained with the non-filtered charcoal-trypsin-casein mixture is produced by the whole amount of trypsin transferable under the conditions observed, and the digestive power of the filtrate is produced by the amount of trypsin combined with the casein before the filtration took place. The process of extraction is finished when filtered and non-filtered specimens give the same effect; the extraction is likewise finished when two samples, obtained by filtering after different intervals of time, show the same digestive power.

In the experiments of which I am going to give an account, the influence of the following factors upon the amount of trypsin extracted by casein from charcoal has been tried :—

1. The temperature.
2. The time of interaction.
3. Amount of water present.
4. Amount of casein.

In all the specimens digested toluol was used as antiseptic, and the effect of the trypsin extracted was determined, as described in previous papers of mine, by estimating the amount of nitrogen in equal volumes of the tannic acid filtrates.[1]

1. *Journ. of Physiol.*, XXXII, p. 390, 1905.

All my experiments were carried out with charcoal-trypsin mixtures in which complete adsorption had been effected. Therefore, all the tryptic effect obtained was due to trypsin which had been transferred from the charcoal to the casein used. According to the investigations referred to, the amount of trypsin transferable is less the larger the amount of charcoal used, the longer the time of interaction between the charcoal and the trypsin before the casein is added, and the higher the temperature for this interaction.[1] Since the trypsin used was not the same in all experiments, and other conditions likewise were varied, only the figures obtained in the same experiment are comparable.

Influence of the Temperature

Experiment I.—The charcoal-trypsin mixture had been kept for twenty-four hours at 37°. Equal volumes of this charcoal-trypsin and of casein solution[2] were mixed and kept at 20° and at 0°, and after the intervals of time recorded the resulting mixtures were filtered at the same temperatures. Then equal volumes of the filtrates were *digested at* 37° for two days. At the same time one specimen which had not been filtered was digested. This specimen, therefore, showed a digestive power corresponding to the amount of trypsin transferable at 37°, and the filtered samples gave the amounts transferred at 20° and at 0°.

The results were :—

	37°	20°	0°
Unfiltered specimen	9·7	—	—
Filtered after ½ hour	—	4·4	2·4
,, ,, 1 ,,	—	4·6	2·45
,, ,, 1½ ,,	—	4·45	—

Therefore the extraction was finished after half an hour, both at 20° and at 0°, and the experiment shows that *more trypsin is transferred the higher the temperature.*

Experiment II.—The charcoal-trypsin was kept at 20° for two days. Then equal charcoal-trypsin-casein mixtures were prepared, kept and filtered at 37° and at 18°. Equal volumes of the filtrates and one unfiltered specimen, were all *digested at* 37° for two days.

	37°	18°
Filtered after ½ hour	5·85	5·3
,, ,, 1 ,,	6·4	5·35
Unfiltered specimen	8·1	—

1. *Bio-Chemical Journal*, I, p. 495, 1906.
2. The solutions used contained 2·5 per cent. of casein in 0·2 per cent. Na_2CO_3 solution.

At 18° the extraction was completed after half an hour, but at 37° it was not finished in one hour, since the total amount of trypsin transferable at 37° is represented by the figure 8·1, obtained from the-unfiltered specimen. The experiment confirms the result of Experiment I as to the influence of the temperature upon the amount of trypsin extracted, and in addition it shows that the extraction comes to an end more speedily at 18° than at 37°.

Influence of the Time of Interaction

Experiment III.—The charcoal-trypsin was kept at 20° for twenty-four hours. Then mixtures were prepared and kept at 20° as follows :—

30 c.c. charcoal-trypsin	+ 150 c.c. cas. sol.	... ...	A
,, ,,	+ 15 c.c. ,,	... ...	B

After the intervals of time recorded, specimens of the mixture were filtered. Of A 30 c.c. filtrate was digested by itself, and of B 7·5 c.c. filtrate was digested together with 22·5 c.c. casein solution. All specimens were digested at 37° for twenty-four hours, and the effect was determined as usual.

	A	B
Filtered at once	9·4 ...	4·6
Filtered after ½ hour	12 ...	5·8

The experiment confirms the observation made in Experiment II, that *the extraction of the trypsin requires some time.* In addition it shows that more trypsin is extracted by the larger amount of casein, but this result does not seem to be quite conclusive since it was not shown in any of the cases that the extraction was finished.

Influence of the Amount of Water Present

Experiment IV.—The charcoal-trypsin was kept at 20° for twenty-four hours, whereupon mixtures were prepared and kept at 20°.

50 charcoal-trypsin	+ 50 casein sol.	... ...	A
,, ,,	,, + 300 H_2O	... ...	B
,, ,,	,, + 500 H_2O	... ...	C

After three hours, when the extraction must have been finished according to Experiments I and II, the mixtures were filtered at 20°, and specimens were prepared for digestion at 37° as follows, the results of the digestion being given at the same time :—

50 filtrate A	+ 250 H_2O + 50 cas. sol.	44·85
200 filtrate B	+ 100 H_2O + 50 cas. sol.	44·0
300 filtrate C	+ 50 cas. sol.	44·05

The ratio between the volumes of A, B, C was 1 : 4 : 6. The digested specimens contained the filtrates in the same proportions and therefore the specimens contained the same amounts of the original charcoal-trypsin and of the casein solution. During the digestion the concentration was the same in all specimens; 50 c.c. casein solution was added in order to increase the effect. The results show, that *the amount of water present during the extraction was without influence.*

Experiment V was carried out with charcoal-trypsin obtained from the same reagents and in the same way as in Experiment IV. Mixtures with casein were made up as follows :—

100 charcoal-trypsin	+ 50 cas. sol.	... ...	A
,, ,,	,,	+ 450 H_2O	B
,, ,,	,,	+ 750 H_2O	C
100 charcoal-trypsin	+ 100 cas. sol.	... ...	D

After three hours at 20° the mixtures were filtered, and specimens made up as follows were digested at 37°, with the results given :—

25 filtrate A	+ 125 H_2O + 50 cas. sol.	27·6
100 filtrate B	+ 50 H_2O + 50 cas. sol.	27·45
150 filtrate C	+ 50 cas. sol.	27·15

The digested specimens contained amounts of the filtrates corresponding to the same amount of the charcoal-trypsin and of the casein. Moreover, the volumes and the amount of casein present were the same during the digestion. Therefore the figures obtained confirm the result obtained in Experiment IV, that *the amount of water exercises no influence.* Since it might be suggested that this result depends upon the casein having extracted the maximum of trypsin, which would not be increased by a larger amount of casein, I tried whether the larger amount of casein present in mixture D had not extracted more trypsin than the casein in the mixture A. For this purpose specimens were made up for digestion as follows, and with the results recorded :—

15 filtrate A	+ 5 cas. sol. + 25 cas. sol.	13·2
20 filtrate D	+ 25 cas. sol.	17·5

15 filtrate A correspond to the same amount of charcoal-trypsin as 20 filtrate D. In order to make the amount of casein the same in both specimens during the digestion, 5 c.c. had to be added to 15 filtrate A. To both, 25 casein sol. was added, in order to increase the effect. The figures obtained show that the larger amount of casein had extracted more trypsin, and therefore the maximum of trypsin extracted had not been reached with the lower amount of casein.

Influence of the Amount of Casein

Experiment VI.—The charcoal-trypsin had been kept at 37°. Mixtures were prepared at 37° as follows :—

50 charcoal-trypsin	+ 250 cas. sol.	... ...	A
,, ,,	+ 100 ,,	... ...	B
,, ,,	+ 50 ,,	... ...	C
,, ,,	+ 10 ,,	... ...	D

The mixtures all contain the same amount of charcoal-trypsin mixture, and since the ratio between the volumes is 60 : 30 : 20 : 12, volumes of the mixtures or of their filtrates, between which the same ratio is valid, must contain trypsin obtained from the same amount of charcoal-trypsin mixture. After 1½ hours at 37° part of the mixtures were filtered and equal volumes of the non-filtered mixtures and of the filtrates were digested at 37° with the following results :—

		Non-filtered		Filtered
60 c.c. A	...	21·55	...	20·1
30 c.c. B	...	15·9	...	15·2
20 c.c. C	...	10·65	...	10·8
12 c.c. D	...	2·55	...	2·45

The figures obtained, being the same for the non-filtered and filtered specimens (except perhaps for mixture A), show that the extraction of the trypsin was finished when the filtration took place.

In order to compare the amounts of trypsin extracted by different amounts of casein, volumes of the filtrates derived from the same amount of charcoal-trypsin mixture must be digested with the same amount of casein. For this purpose, specimens prepared as follows were digested, the results of the digestion being given at the same time :—

60 c.c. filtrate	A	20·1
30 c.c. ,,	B + 30 c.c. cas. sol.	17·8
20 c.c. ,,	C + 40 c.c. ,, ...	16·5
12 c.c. ,,	D + 48 c.c. ,, ...	8·95

The figures show that the amount of trypsin extracted increases with the amount of casein used for the extraction. Unfortunately, the amount of casein present during the digestion was not sufficient to make the results proportional to the amounts of trypsin present, and therefore no further definite conclusions can be drawn from this experiment. It is, however, obvious that the amounts of trypsin extracted were not nearly proportional to the amounts of casein used, the ratio between the amounts of casein being 25 : 10 : 5 : 1.

This being so, one must expect that for very large amounts of casein the amount of trypsin extracted should become independent of the amount of casein used for the extraction. This was the case in the following experiment :—

Experiment VII.—The charcoal-trypsin had been kept at 37°, and the following mixtures were prepared and kept at 37°.

10 c.c. charcoal-trypsin	+ 50 c.c. cas. sol.	...	...	... A
,, ,,	+ 100 ,,	+ 10 H_2O	...	... B
,, ,,	+ 200 ,,	+ 30 H_2O	...	... C

After one hour at 37° the mixtures were filtered, and volumes of the filtrates derived from the same amount of charcoal-trypsin were digested with the same amount of casein as follows :—

20 c.c. filtrate	A	+ 50 c.c. cas.	+ 10 H_2O	...	...	23·5
40 ,,	B	+ 33·3 ,,	+ 6·7 ,,	...	...	23·5
80 ,,	C	...	...	...	...	23·6

That the extraction was finished when the samples were filtered was ascertained by another experiment. A mixture of 30 c.c. charcoal trypsin + 300 c.c. casein was prepared and kept as above. Then 100 c.c. unfiltered mixture and 100 c.c. filtrate were digested :—

Unfiltered mixture	...	...	65·8
Filtrate	...	...	... 64·4[1]

Discussion of the Results

The above experiments give, no doubt, a very strong support to the view that the proteins combine with trypsin before they are broken up by the same. As the experiments were arranged, the casein was made to extract trypsin which had been previously adsorbed by charcoal. During the process of extraction, therefore, the trypsin was acted upon by two substances endowed with the power of taking it up—charcoal and casein. The final distribution of casein between the charcoal and the trypsin was reached in, as a rule, less than half an hour after the casein had been added to the charcol-trypsin mixture. Both charcoal and casein take up trypsin in some proportion to the amount of substances present, and therefore the final distribution of the trypsin will be the effect of some kind of mass action

1. With regard to the above experiments, it may be mentioned that it has been found to be very difficult completely to remove charcoal from a fluid that contains casein. Even after repeated filtration traces of charcoal occur in the filtrate. Evidently these traces can be of no influence, in case the extraction was finished when the filtration took place and if at the same time the following digestion is carried out at the same temperature as the extraction and filtration (Experiment VI, VII). In other cases the figures obtained from the filtered specimens might be a trace too high, which would, however, in no instance affect the conclusions drawn. The amount of digestion taking place before and during the filtration, being the same or approximately the same for filtered and non-filtered specimens, does not influence the results.

On the other hand, the effect of this mass action can not be the same as that of Guldberg-Waage's law for real solutions, because in the case of real solutions the combinations formed are reversible and, therefore, the final equilibrium will be the same independently of the way in which it has been arrived at.

In the case of charcoal and trypsin, on the contrary, the trypsin adsorbed by degrees becomes fixed to the charcoal and only a small portion of it can be extracted by added casein, less being extracted the longer the time and the higher the temperature for the interaction between the charcoal and trypsin before the casein is added.[1] The final distribution of the trypsin, therefore, depends very much upon the way in which it has been arrived at.

Summary

1. Casein is capable of extracting trypsin adsorbed by charcoal. The extraction requires some time to be finished (Experiment III). Sooner or later (as a rule in less than half an hour at 20°) the extraction comes to an end as borne out by the fact that a filtered specimen gives the same tryptic effect as the non-filtered material (Experiments VI, VII). The end of the extraction can also be recognised by the fact that specimens filtered after different intervals of time contain the same amount of trypsin (Experiments I, II).

2. The final amount of trypsin extracted is larger the higher the temperature (Experiments I, II).

3. For small amounts of casein the final amount of trypsin extracted increases with the amount of casein (Experiment VI). For large amounts of casein the amount of trypsin extracted is independent of variations in the amount of casein (Experiment VII).

4. The amount of water present is without influence upon the final amount of trypsin extracted (Experiments IV, V). This rule holds good even for small amounts of casein (Experiment V).

The results support the view that proteins combine with trypsin before they are broken up by the same.

1. *Bio-Chemical Journal*, I, 489, 1906.

A CASE OF SPECIFIC ADSORPTION OF ENZYMES

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(*Received December* 31*st*, 1906)

In this journal I have recently given an account of experiments on the anti-tryptic action of charcoal.[1] The process by which trypsin was rendered inactive could be divided into two consecutive stages: the mere adsorption or taking up of the trypsin by the charcoal, and the fixation of the same. The amount of trypsin adsorbed could be represented by the difference between the digestive power of the trypsin solution before the treatment with charcoal and that of the filtrate after the treatment. In addition it was found that the action of charcoal was very much like that of the tryptic antibody in the serum, and it was suggested that the effect was brought about in the same way in both cases.

I have extended these investigations, as far as the mere adsorption is concerned, to the proteolytic enzymes of the spleen, at the same time trying the influence of another solid substance, namely, kieselguhr. That kieselguhr may be capable of taking up enzymes, has been shown by Dauwe.[2] Previously I have found that the spleen of the ox contains at least two proteolytic enzymes, the one acting principally in an alkaline medium (α-protease) and the other in an acid (β-protease).[3] In addition, the spleen (and the serum) contains an antibody which decidedly checks the digestion with alkali, whilst it has practically no effect upon the digestion with acid.[4] Since this antibody is destroyed by the action of weak acetic acid, the spleen substance shows a stronger effect with alkali after previous treatment with acetic acid than it does without treatment with acid. After previous treatment with acid the spleen-pulp gave,

1. *Bio-Chemical Journ.*, I, 484, 1906.
2. *Hofmeisters Beiträge*, Vol. VI, p. 427, 1906.
3. *Journ. of Physiol.*, XXX, p. 155, 1903.
4. Hedin: 'An explanation of the influence of acid and alkali on the autolysis of organs' (*Hammarstens Festschrift*, Upsala, 1906).

roughly, the same effect with alkali as with acid. On trying the effect of juice pressed from spleen substance after grinding with silver sand *and mixing with kieselguhr*, it was, however, found that even after previous treatment of the juice with acid the effect was much stronger with acid than it was with alkali. This I suggested to be due to the antibody present neutralising the α-protease more readily in the liquid juice than in the semi-solid spleen pulp. Lately I have found that there is another factor which tends to produce the same result. As shown by the following experiments, kieselguhr adsorbs the α-protease more readily than it does the β-protease. Since a very large amount of kieselguhr has been used in preparing the spleen-juice, very much of the α-protease has no doubt been detained in the press-cake, adsorbed by the kieselguhr, whilst the β-protease has passed almost entirely into the filtrate.

A mixture of the two enzymes was prepared by digesting the spleen with 0·1 per cent. acetic acid at 37° for two days. Then the fluid was filtered off, and in order to obtain a more concentrated enzyme solution, the filtrate was precipitated by saturation with Am_2SO_4. The salt was removed from the precipitate by dialysis, and the resulting solution was used for digestion.

In order to be able to estimate roughly the amount of enzymes adsorbed, I have tried whether the enzymes, like trypsin, give results proportional to the amounts of enzyme present when digested with a large amount of casein.

200 c.c. of 2·5 per cent. casein in 0·25 per cent. Na_2CO_3 solution was taken to each sample. In the case of the β-protease, acid reaction was effected by adding 5 c.c. of 20 per cent. acetic acid to each specimen. The estimation of the digestion which had taken place was carried out by determining the nitrogen in equal volumes of the tannic acid filtrates, as described in previous papers. The figures given represent cubic centimetres of decinormal acid required for neutralising the ammonia obtained.

	With alkali	With acid
10 c.c. Protease sol. + 10 c.c. H_2O	12·2	12·9
20 c.c. ,, ...	28·85	24·1

In accordance with what I have found before,[1] the effect with alkali shows a tendency to increase more rapidly than the amount of enzyme. On the other hand, the effect with acid is very nearly proportional to the amount of enzyme.

The charcoal used was animal charcoal, prepared from bones and purified with HCl. The kieselguhr contained only traces of substances soluble in water and the watery extract of the same gave a perfectly neutral reaction.

In order to effect adsorption of the enzymes equal volumes of enzyme mixtures, prepared as above, were heated with equal weights of charcoal and of kieselguhr *in a neutral medium* at 37° for about two hours. In the same way a control was heated without any adsorbing substance. Then the charcoal and the kieselguhr were filtered off and equal volumes of the control and of the filtrates were taken for digestion with casein, as above.

The results obtained with two different enzyme mixtures were as follows :—

	With alkali	With acid	Effect with acid / Effect with alkali
I			
Without adsorption	22·8	31	1·36
After adsorption by charcoal ...	4·2	6·05	1·44
„ „ kieselguhr ...	9·3	27·7	3·0
II			
Without adsorption	11·5	19·6	1·7
After adsorption by charcoal ...	3·6	6·3	1·75
„ „ kieselguhr ...	6·0	17·7	2·95

Since the same weight of charcoal and of kieselguhr was used, charcoal in this case exercises a more powerful adsorption than does kieselguhr. Both enzymes are adsorbed equally readily by charcoal, the ratio between the effect with acid and with alkali being the same after the adsorption as before.

On the other hand, the effect of the kieselguhr is different inasmuch as a pronounced reduction in the digestion with alkali takes

1. *Hammarstens Festschrift.*

place, whilst the digestion with acid is very little affected. In fact the slight diminution in the digestion with acid might depend upon adsorption of the α-protease, since this enzyme, although acting principally with alkali, might be able to exercise some slight action with acid too. Kieselguhr therefore evidently adsorbs a considerable amount of α-protease, whilst it must be left undecided whether a slight adsorption of the β-protease takes place or none at all.

In the above experiments it was not proved that the process of adsorption had come to an end when the casein was added, and therefore the results obtained do not necessarily correspond to the final distribution of the enzymes. The results show that the *rate of adsorption* by charcoal is approximately the same for both enzymes, whilst the *rate of adsorption* by kieselguhr is higher for the α-protease than for the β-protease.

The experiments bear out the fact that the adsorption of enzymes can be, to a large degree, specific.

In this connection it is interesting to note similar experiments carried out by Bayliss,[1] who found that filter paper adsorbs congo-red and acid fuchsin in the same proportions, like charcoal does the two spleen enzymes, whereas gelatine adsorbs a larger proportion of congo-red than it does of acid fuchsin, thus forming a parallel to the kieselguhr in the case of the spleen enzymes.

The amounts of spleen enzymes adsorbed by charcoal in the above experiments are rather insignificant, as compared with the enormous amounts of trypsin adsorbed by the same amount of charcoal in my investigations on the adsorption of this enzyme.[2] On the whole, charcoal seems to possess quite an exceptional aptitude for adsorbing trypsin. This might, perhaps, depend upon some specific relationship.

It must, however, be borne in mind that the trypsin solutions used in my experiments were nearly free from proteins, whilst the solutions of the spleen enzymes held large amounts of proteins, which could not be digested away lest the enzymes were too much weakened. In the case of trypsin, it was found that the addition of

1. *This Journal*, I, p. 212, 1906.
2. *This Journal*, I, p. 484, 1906.

casein prevents some trypsin from being adsorbed by the charcoal; in fact, casein is capable of extracting trypsin already adsorbed by the charcoal. Therefore the large amounts of proteins present might account for the spleen enzymes being less readily adsorbed by charcoal than trypsin. On the other hand, the different behaviour of the two spleen enzymes to kieselguhr cannot be accounted for by a different amount of proteins, because they were acted upon in the same fluid.

In conclusion, I wish to call attention to rather an important conclusion which can be drawn from the above experiments, namely, that juices prepared with kieselguhr, as proposed by Buchner in the case of yeast, do not necessarily contain the enzymes in the same proportions as in the cells used. The enzymes might be adsorbed by the kieselguhr, or perhaps by the solid remains of the cells, and consequently some of them might, perhaps, be completely retained in the press-cake.

Summary

The α and β-protease in the spleen of the ox, are adsorbed in the same proportions by charcoal, whilst kieselguhr adsorbs the α-protease much more readily than the β-protease, and it seems doubtful whether the latter be adsorbed at all. This fact might account for the observation that juice pressed from the spleen after grinding and mixing with kieselguhr contains relatively less α-protease than does the organ itself.

[*Reprinted from the Journal of Physiology, Vol. XXXV. Nos.* 1 & 2, *December* 29, 1906.]

ON DIURNAL AND NOCTURNAL VARIATIONS IN THE EXCRETION OF URIC ACID. By J. B. LEATHES.

In studying certain aspects of nitrogenous metabolism the following procedure was adopted. A diet of sufficient caloric value was taken, which was distributed over six meals, all of which were identically the same, taken at regular intervals of three hours from seven in the morning till ten at night. This diet contained no purine derivatives in any form. The regular distribution of such a number of small meals was designed to eliminate as far as possible the irregularities in excretion which are caused by the digestion and assimilation of nitrogenous foodstuffs. And with the same object in view, not only were diuretic drinks such as tea and coffee omitted entirely from the diet, but the volume of fluid taken at each meal was the same throughout, as well as the amount and nature of the dissolved or solid foods. Between the hours of ten p.m. and seven a.m. it is true there was no intake either of solids or liquids: for the greater part of this time was taken up in sleep. But for eighteen hours out of the twenty-four the conditions were in many important respects the same.

The urine was collected at the hours at which the meals were taken and another sample was obtained, or sometimes two, during the night. Each sample was measured and analysed, and from the analytical results the rate was calculated at which each of the substances for which the data were obtained had been excreted in each of the periods of the day. In that way curves could be drawn indicating the variations in these rates; and it is these curves that sum up the substance of this paper.

The analyses were carried out as follows: (1) two samples were taken for the estimation of the total nitrogen by Kjeldahl's method.

(2) The creatinine was estimated colorimetrically by a method which in essentials is that described by Folin[1]. Both these determinations were carried out on the same day as, or at any rate within twenty-four hours of, the collection of the samples.

[1] Folin. *Zeits. für physiol. Chem.* XLI. p. 223. 1904.

(3) 50 c.c. or when possible 100 or 150 c.c. were measured off into stoppered bottles containing 10, 20 or 30 grs. of ammonium chloride and put aside for the estimation of uric acid at the end of the experiment. This was then done according to the modification of Hopkins' method suggested by Wörner[1].

The first experiment began on the 11th of August and ended on the 23rd. During this time the food taken each day consisted of 2 quarts (2·28 litres) of milk, obtained from a large dairy supplying a product of nearly constant composition, 600 g. of bread and 110 g. of butter. The heat equivalent of this diet was calculated to be about 3500 cal. or just over 50 cal. per kg. of bodyweight. The daily nitrogen intake was about 21·5 g. and the daily output in the urine from the fourth day on when the variations began to be insignificant was about 19 g. on an average. The total nitrogen and creatinine was estimated in the urine of each period of all these days, the uric acid similarly only during the last week. In the following table the figures, calculated from the results of the analyses, represent the amount of uric acid excreted in each hour of the period ending at the time indicated at the top of the column.

TABLE I.

Observed rates of uric acid excretion, in mg. per hour, during the periods ending at the hour of:

	3 a.m.[1]	7 a.m.	10 a.m.	1 p.m.	4 p.m.	7 p.m.	10 p.m.
Aug. 16	—	—	—	39·6	35·5	—	20·4
17	24·7	23·5	25·7 (some loss)	37·2	30·3	26·6	27·2
18	19·0	25·5	35·6	28·1	31·0	29·3	24·9
19	18·5	23·0	34·7	30·7	25·7	27·6	20·6
20	19·1	19·0	31·9	35·9	—	25·4	25·1
21	21·0	21·0	29·6	37·0	34·7	26·5	19·9
22	21·8	22·2	—	25·5	27·2	26·9	23·9
23	22·9	23·4	26·6	26·4	—	—	—
Calculated averages from the above observed rates:							
	21·0	22·5	31·3	32·5	30·7	27·0	23·1

[1] The hour at which the samples in this column were collected varied on the different days.

In the second experiment, which began on Sept. 13 and ended on Sept. 26, the food consisted of biscuits (Huntley and Palmer's plain arrowroot), butter and water. Of the biscuits six were taken at each meal, the weight of the daily ration being 540 g. with 36·5 g. of moisture. Three samples dried to constant weight contained 1·00, 1·15 and 0·97 % of nitrogen, which gives a daily intake of nitrogen of about 5 g., or a little

[1] Wörner. *Ibid.* XXIX. p. 70. 1900.

over 30 g. of proteid. The amount of butter taken in the day was the same as in the first experiment. This diet therefore contained less than a quarter of the amount of nitrogen contained in the diet of the earlier experiment, and in fact nitrogenous equilibrium was not attained on it. The heat equivalent also was very considerably lower than in the case of the earlier diet. Nevertheless the loss of weight was inconsiderable. The urine was collected and analysed as before and the following table is constructed similarly to the last.

TABLE II.

Observed rates of uric acid excretion, in mg. per hour, during the periods ending at the hour of:

	3 a.m.	7 a.m.	10 a.m.	1 p.m.	4 p.m.	7 p.m.	10 p.m.
Sept. 17	—	—	—	24·3	18·1	24·3	21·4
18	8·0	13·7	20·4	23·0	20·3	18·1	14·1
19	14·1	15·6	19·4	25·6	23·9	19·5	14·5
20	9·5	12·7	23·0	27·3	23·3	18·0	14·9
21	12·1	17·9	22·2	23·7	24·8	26·0	23·5
22	8·1	18·0	19·3	17·8	17·0	20·1	17·3
23	8·5	8·3	11·2	25·1	18·9	17·6	17·5
24	15·2	18·7	22·6	27·7	22·7	—	—
Calculated averages from the above observed rates:							
	10·8	15·0	19·7	24·3	21·1	20·5	17·6

The average rates of uric acid excretion in the corresponding periods of the different days, which are given in the last line of Tables I and II, are laid out in the form of curves in Fig. 1, in which the upper curve is that given by the results of Exp. I and the lower by those of Exp. II. In order to show more clearly the form of the curve of diurnal variations, it is extended over a period of 48 hours. The curves for the creatinine, and for the total amount of nitrogen excreted, calculated as averages in a similar way, are given for comparison, the former above the uric acid curves in Fig. 1, the latter in Fig. 2.

It will be noticed that in the subject of these experiments at any rate there is a remarkable difference between the rate of uric acid excretion at different times in the day. It is very much slower at night than by day. The maximum is in the morning from ten to one. After this there is a steady decline till the minimum in the earlier part of the night, when the rate of excretion on the low nitrogen diet is less than half that found in the forenoon. In the first waking hours from seven to ten the rate is considerably increased, and in fact there is a fairly definite tendency for the rate to rise in the latter part of the night.

The very close agreement in the general form of the two curves obtained from the two experiments clearly shows that this variation in the different periods of the day is not merely accidental, but a general characteristic of the functions concerned. To prove however whether

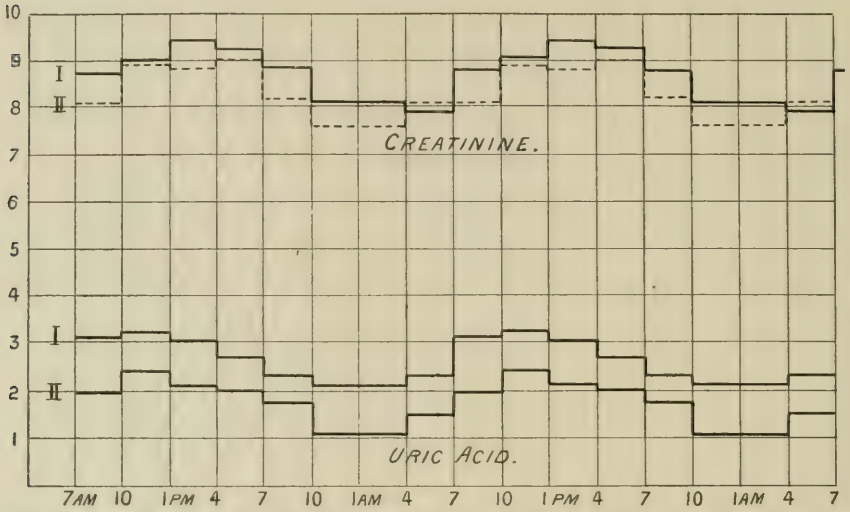

Fig. 1. In the figure the ordinates give the average rate of excretion of uric acid and creatinine in centigrams per hour for each of the periods indicated on the abscissa. The Roman numbers in the left-hand column refer to the number of the experiment.

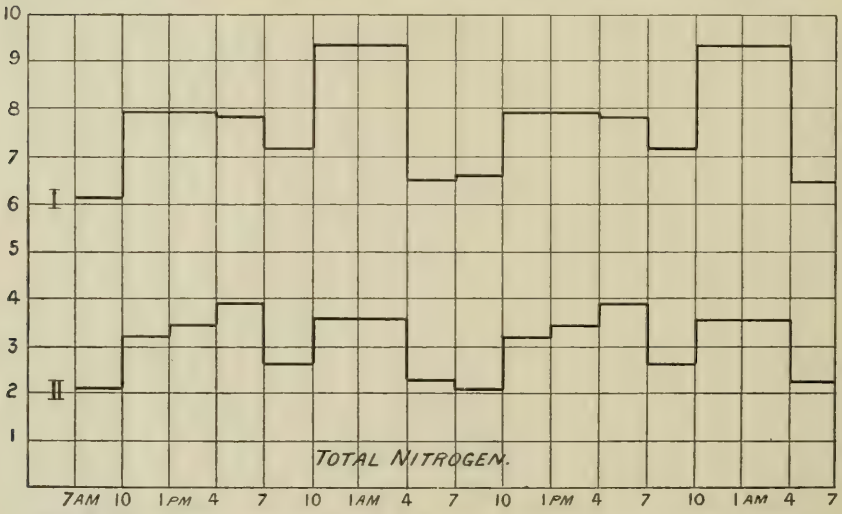

Fig. 2. The figure constructed like Fig. 1 gives for the two experiments the rate of excretion of nitrogen in all forms expressed in diagrams per hour.

it is due to idiosyncrasy, or whether it indicates a physiological law of more general interest, experiments are being carried out on other subjects. These, together with experiments bearing on the interpretation of the phenomenon will form the subject of a second paper which is in preparation, and for which will be reserved the full discussion of this and of other facts which are summed up in the curves of the rates of excretion of creatinine and total nitrogen which are also given in Table III.

For the present it will be sufficient to point out that the diminution of the rate of excretion of uric acid during the night, like the similar though less striking diminution in the rate of excretion of creatinine, cannot be explained as simply the result of diminished renal activity during sleep. For the accompanying curves indicating the variations in the rate of total nitrogen excretion show that the kidneys, on a full nitrogen diet at any rate, are more active during sleep than during the waking hours of the day, and even on the low nitrogen diet of the second experiment are almost at their maximum activity in the night. The low output of uric acid and creatinine during the night must be the expression of a diminished functional activity in some other organ or organs than the kidney. Now does it seem probable for the same reason that the explanation is to be found in diminished blood-pressure. The experiments now being carried out with a view to identifying the functions associated with the output of uric acid will be described shortly.

One other point may be noted here : the results of the experiments are in agreement with those of Folin in giving a lower absolute uric acid value on a low nitrogen diet, although the percentage of the total nitrogen excretion formed by the nitrogen of the uric acid is increased. The opposite result obtained by Burian and Schur, which led them to enunciate the law of constant uric acid excretion on a diet free from purine derivatives, has had evidence advanced in support of it by Walker Hall and others, and before Folin's results were published had found general acceptance. The divergence of the evidence on this point will be explained when it is possible to give a satisfactory account of the significance of uric acid among the nitrogenous excreta on such diets.

Conclusions.

1. In these experiments it was found that, when a uniform purine-free diet is taken, the excretion of uric acid is most active in the early waking hours and very much less active at night.

2. This is not a sign of variations in renal activity for the excretion of nitrogenous substances in general is as high during the night as during the day, or even higher.

3. It is a sign rather that there are corresponding variations in the formation of uric acid in the body at different periods in the day.

4. Similar though less marked variations were found in the rate at which creatinine is excreted.

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ON THE RELATION OF THE ANTITOXIN TO THE GLOBULIN-CONTENT OF THE BLOOD SERUM DURING DIPHTHERIA IMMUNISATION.

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Within recent years a considerable amount of attention has been directed to the subject of the relationships subsisting between the proteids of the blood serum and the antibodies elaborated therein by various methods of immunisation.

One result of such study has been to give an impetus to the more accurate differentiation of the several proteid constituents regarded as bodies possessing not only definite physical and chemical properties, but also what we, with our present knowledge, may call functional affinities.

The investigations recorded in the present paper, dealing with the quantitative relations of the serum globulins during the process of immunisation with diphtheria-toxin, were instigated by the fact that, hitherto, with the exception of the work of Hiss and Atkinson (1901) to be later referred to, only isolated observations have been made of the globulin variations in relation to potency.

It was felt that the only satisfactory method of procuring reliable data on the globulin-antitoxin question was to institute frequent quantitative estimations throughout the whole period of immunisation of individual horses or goats and not at random intervals in their history as antitoxin-producers.

For some considerable time it has been a well-established fact that antitoxin is precipitable from serum by any precipitants which throw down the globulins, but more recent research has shown that the term "globulin" comprises two or more bodies having different salt precipitation limits as well as different antitoxin-contents.

The history of this subject, dealing with the differentiation of the globulins and their relation to the antitoxic substance, is so important in connexion with the observations to be presently recorded, that brief reference must be made to the literature. The work of Marcus (1899), following at long interval after the preliminary observations of Burckhardt (1883), went to show that the hitherto accepted characteristic of serum-globulin in contradistinction to serum-albumin, viz. its insolubility in salt-free water was untenable because, after precipitation by dialysis of the typical insoluble globulin, there remained in solution a relatively much larger quantity of a proteid body, presenting no essential differences from the first either in chemical reaction, coagulation temperature, or elemental constitution.

Consequently, according to Marcus, one must either give up insolubility in water as a characteristic property of globulin or consider the soluble globulin as constituting a new proteid group.

So far, it was impossible by fractional salt precipitation to isolate bodies corresponding to these soluble and insoluble globulins.

Before Marcus' work, attempts had been made to determine the chemical nature of diphtheria-antitoxin and its relation to the serum-proteids.

Thus Aronson (1893) found that the globulin precipitated by dialysis was potent as well as the globulin-free filtrate, while Dieudonné (1897), by magnesium sulphate precipitation, obtained the greater part of the antitoxic substance in the precipitate and only a small part in the globulin-free filtrate.

Brodie (1897) was the first to show that diphtheria-antitoxin was completely precipitated from a solution by any means which removed the globulins. He also attempted a fractional precipitation of serum with ammonium sulphate up to half-saturation, thus separating four successive portions, all of which were found to contain antitoxin in equal amount. That the mode of preparation of the globulin was of importance was shown by Belfanti and Carbone (1898), who found that the antitoxin was carried down along with the globulins from ammonium or magnesium sulphate precipitation but not with the precipitate obtained by acetic acid.

In the course of an attempt to follow up Brieger and Boer's work on the preparation of a proteid-free antitoxin, Freund and Sternberg (1899) discovered that by addition of a solution of potash-alum to serum (to one volume serum, one-third volume of a 5 % solution of potash-alum) all the antitoxin remained in the filtrate. In fact, by this

precipitant the albumin was removed first and a filtrate left which, according to Freund and Sternberg, contained practically all the globulin and consequently all the antitoxin. Further, by precipitating this globulin filtrate with ammonium sulphate, it was possible to obtain in small bulk the globulin-content of a large quantity of serum. No further light, however, could be thrown by these investigators on the chemical nature of antitoxin. All that could be said was that the antitoxic substance was precipitated along with the globulins.

The method of treating serum with potash-alum in order to get rid of albumin was employed by Seng (1899), who dealt with the question whether the sera of normal and immune animals presented differences in the amount and nature of their proteid constituents. The "soluble" and the "insoluble" globulin fractions were separately estimated, and the total proteid determined by Kjeldahl.

From the few isolated estimations on horses immune to diphtheria-toxin, no conclusive facts could be elicited as to the relative quantities of total globulin, albumin, and insoluble globulin. Though no quantitative differences could be found between the "soluble" globulin-content of normal and antitoxic serum, certain slight differences in coagulation temperature and specific rotation were made out. It was further determined that prolonged dialysis of the potash-alum filtrate until no reaction to chlorine, ammonia, or sulphuric acid remained, rendered only a very small fraction of the globulin insoluble, viz. 1 : 23 to 1 : 11. All the antitoxin was recoverable from the solution. These latter experiments of Seng threw light on the rather discordant results of earlier writers. Thus the result obtained by Belfanti and Carbone that the antitoxin falls out with the globulins when precipitation is effected by magnesium or ammonium sulphate, but not by acetic acid or carbon dioxide, is explained by the fact that simple neutralization of the serum by acetic acid or carbon dioxide precipitates only the "insoluble" globulin which contains none of the antitoxin. So also the statement by Dieudonné that "reines globulin" precipitated by carbon dioxide or by dialysis contained no antitoxin is explained on similar grounds.

A notable advance was made by Hiss and Atkinson (1901), who estimated the globulin-content of the serum of a large number of horses at different stages of immunisation against diphtheria-toxin. The globulin was precipitated by the magnesium sulphate method. The total proteid was obtained by heat coagulation and the albumin estimated by difference. As a rule not more than two globulin estimations were made on each horse, one before the commencement

of immunisation and the other at some stage in the process. When the potency had reached a level of 300 units or over, the globulin was invariably found to have risen, sometimes to double its original value. In one horse five globulin estimations were made during immunisation, the figures (obtained from 10 c.c. serum) being as follows:

Normal	400 units	600 units	650 units	1200 units
·3235	·4743	·5116	·5934	·8987

A progressive increase in globulin was thus evident as the potency rose. While the globulin rose the albumin fraction progressively diminished. It appeared generally that a low potency coincided with a low globulin-content, but it was impossible to take the absolute amount of globulin as an index of the antitoxin-content of the serum.

Within the last few years important contributions to our knowledge of the globulins have emanated from the Hofmeister school. Fuld and Spiro (1900) determined that by fractional precipitation of serum with ammonium sulphate two globulin fractions could be obtained, one precipitable by 28–33 % saturation and the other only by 34–46 % saturation. To the former fraction precipitable by one-third saturation, Hofmeister gave the name "euglobulin," and to the latter precipitable only by half-saturation, the name "pseudoglobulin." Included in the euglobulin fraction is the fibrinoglobulin, which Reye (1898) had already found to be precipitated by 21·5 % saturation with ammonium sulphate. The interesting fact was recorded that these three fractions differed with regard to their influence on milk coagulation. The fibrinoglobulin had no constant action either in producing or preventing coagulation. The euglobulin had decidedly the power of coagulating milk, while the pseudoglobulin had a more or less pronounced inhibitory action on milk coagulation.

The relation of diphtheria-antitoxin to the three globulin fractions precipitable by ammonium sulphate up to half-saturation was investigated at great length by Pick (1902), who found that neither the fibrinoglobulin nor the euglobulin of horse serum contained the antitoxin. The pseudoglobulin, on the other hand, contained all the antitoxin. On the other hand, when goat serum was salted out in the same way and the fractions tested as to their antitoxin-content, it was found that the euglobulin contained the antitoxic substance.

Freund and Joachim (1902) repeated much of Spiro's work, and were unable to identify completely the euglobulin and pseudoglobulin with the "insoluble" and "soluble" globulin obtained by dialysis,

acetic acid or sodium chloride. It was determined that both the euglobulin and the pseudoglobulin could again be split up into two portions, one of which was soluble and the other insoluble in water. They accordingly recommended the following as a more accurate classification of the globulins:

Fraction obtained by one-third saturation	α.	Insoluble in H_2O.	Paraeuglobulin.
	β.	Soluble in H_2O.	Euglobulin.
Fraction obtained by half-saturation	α.	Insoluble in H_2O.	Parapseudoglobulin.
	β.	Soluble in H_2O.	Pseudoglobulin.

There is no doubt that the division into euglobulin and pseudoglobulin is a more or less artificial one from the purely chemical point of view as Hammarsten maintains, but the remarkable differences exhibited by them in their capacity as antibody-carriers proclaim a real duality and render it highly essential that we should retain this somewhat arbitrary mode of separation.

Porges and Spiro (1903) admitted the correctness of Freund and Joachim's statement that the eu- and pseudoglobulin fractions did not represent exactly the sum total of the insoluble and soluble globulins respectively. In the same year Joachim (1903) recorded a few quantitative estimations of the globulin fractions in a horse immune to diphtheria-toxin. The estimations were made on two occasions, one before the commencement of immunisation and the other three months later, when the serum had reached a potency of 500 units. He found no essential increase of the total proteid contrary to what had previously been noted by Butjagin (1902) and Szontagh and Wellman (1898). The total globulin was, however, markedly increased at the expense of the albumin, but contrary to expectation the rise affected solely the euglobulin fraction which contained none of the antitoxin. His figures were:

Before immunisation,

Eugl. : Pseudogl. : Alb. :: 12·74 : 37·04 : 50·21.

After immunisation,

Eugl. : Pseudogl. : Alb. :: 26·21 : 36·74 : 37·05.

This remarkable result we shall discuss later.

The question of globulin variations in other experimental infections has been approached by Langstein and Mayer (1904), who showed that in rabbits immunised against typhoid, pneumococcus, sheptococcus, dysentery, cholera, and swine erysipelas the serum-globulin rose while

the albumin diminished. It was determined also by Mayer (1905) that the serum of dogs infected with *Trypanosoma* showed an increase of globulin and a diminution of albumin, although the total proteid was not markedly affected.

The objections which several authors have recently brought forward against the view that globulin-change is a necessary concomitant of the elaboration of antibodies will be discussed in the course of this paper in the light of our own observations.

Description of Technique and Mode of Investigation.

Though the main object of the present investigation was to ascertain by examination at frequent intervals whether any definite relationship could be traced during immunisation between the rise in potency and the globulin variations, it appeared desirable also to determine how far Pick's statements as to the antitoxin-contents of the various globulin fractions held good in the horse and goat.

For the first two series of investigations a horse (Plug) and a goat (Mephistopheles) were employed. The former was treated by the usual method which obtains at this institute, and previous to the commencement of immunisation estimations were made of the normal globulin and normal antitoxin present in the blood serum.

Half-saturation with ammonium sulphate was always employed to separate the total globulin from the albumin. The precipitate was redissolved in water and again treated with ammonium sulphate. After filtration the precipitate was again dissolved and coagulated by heat. The coagulated globulin was collected on a weighed filter paper, thoroughly washed with hot water and then dried in vacuo over sulphuric acid for several days. After a final dessication in an air-bath at 80° it was weighed.

The filtrates containing the albumin were also coagulated and weighed in a similar manner. In estimating the total proteid a fixed quantity of serum, 10 c.c., was diluted by addition of 190 c.c. of distilled water, and carefully acidified with acetic acid. It was then coagulated by heat and the precipitate dried and weighed. Where complete estimations of the euglobulin and pseudoglobulin were made, as well as of the total globulin and total proteid, three quantities of serum (each 10 c.c.) were used. With one quantity the total coagulable proteids were estimated, with another the total globulin and total albumin, and with the third quantity the euglobulin was obtained by third-saturation

with ammonium sulphate, the filtrates being employed for the estimation of the combined pseudoglobulin and albumin. The difference between this last and the albumin previously estimated, gave the amount of pseudoglobulin.

The potency of the serum was estimated in Ehrlich-Behring units by the usual methods practised in this Institute.

Immunisation of Horse (Plug).

Immunisation was commenced on 17 Nov. 1905, with an inoculation of ·01 c.c. toxin. Inoculations were made thereafter every third or fourth day, provided all local swelling had disappeared, each successive dose being double the preceding one.

In the following table (p. 72) are indicated the toxin-doses and the potency of the serum on the different dates.

According to the practice at this Institute the horses are bled nine days after they have received the final dose of 1000 c.c. toxin. The potency of the serum when tested at this time, *i.e.* after the first bleeding varies as is well known in different horses quite apart from the methods of immunisation employed. In our experience here the great majority of the horses attain a potency of 600 units and over, at the first bleeding, but there is always a small percentage of horses which do not reach 300 units. These latter are of course discarded so far as their employment for the production of diphtheria-antitoxin is concerned.

From the following table it will be seen that unfortunately for the object of our experiment, this horse proved unsuitable for the production of high grade antitoxin. Our hopes that the serum might attain a high antitoxin-content so that a suitable comparison might be made between this and the globulin-content were disappointed.

From the purely theoretical point of view, however, the facts brought by the immunisation of this refractory horse, present numerous points of interest and enable one to institute some comparison between the reaction to diphtheria-toxin of such horses and the more responsive animals.

On the 10th day after the first dose of 1000 c.c. toxin (2nd Feb. '06), (see Table I. and Chart I.), the serum had an antitoxin-content of only 180—200 units per c.c. One litre of blood was drawn and a further dose of 1000 c.c. given on 5th Feb. On the 9th day thereafter (14 Feb.) the potency had risen slightly to 240—250 units. On the 19th Feb., 22nd Feb., and 26th Feb. further doses of 500 c.c., 700 c.c., and 1000 c.c.

respectively, were administered with little appreciable effect on the potency.

TABLE I.

Date	Toxin-dose	Potency (in units)
Nov. 10 '05	—	$\frac{1}{20}$—$\frac{1}{15}$ per c.c.
17	·01	—
20	·02	$\frac{1}{16}$—$\frac{1}{4}$
23	·04	$<\frac{1}{8}$
27	·1	1—2
30	·2	2—4
Dec. 4	·5	2—4
7	1·0	<6
11	2	4—6
15	4	—
19	8	8—12
22	15	8—12
26	30	12—16
29	60	12—20
Jan. 1 '06	120	<20
4	250	20—28
8	500	40—50
12	500 (new tox.)	60—80
16	800	80—100
19	—	110
23	1000	110—120
26	—	140—160
31	—	160—180
Feb. 2	—	180—200
5	1000	—
9	—	<200
14	—	240—250
19	500	—
22	700	—
26	1000	<240
Mar. 7	—	250—300
12	1000 intraven.	—
21	—	<200

Thus on 7th Mar. the serum had only between 250 and 300 units per c.c. A final attempt to raise the potency was made on 12th Mar. when 1000 c.c. toxin were injected intravenously by the jugular vein. The animal suffered no bad effects from the injection, but after a lapse of 10 days the potency instead of being raised had actually fallen below 200 units per c.c. In fact this latter experiment is in complete accord with a previous one made by Dr Dean and which goes to show that the intravenous injection of diphtheria-toxin has little or no effect in raising the potency in a horse whose antitoxin-content is falling.

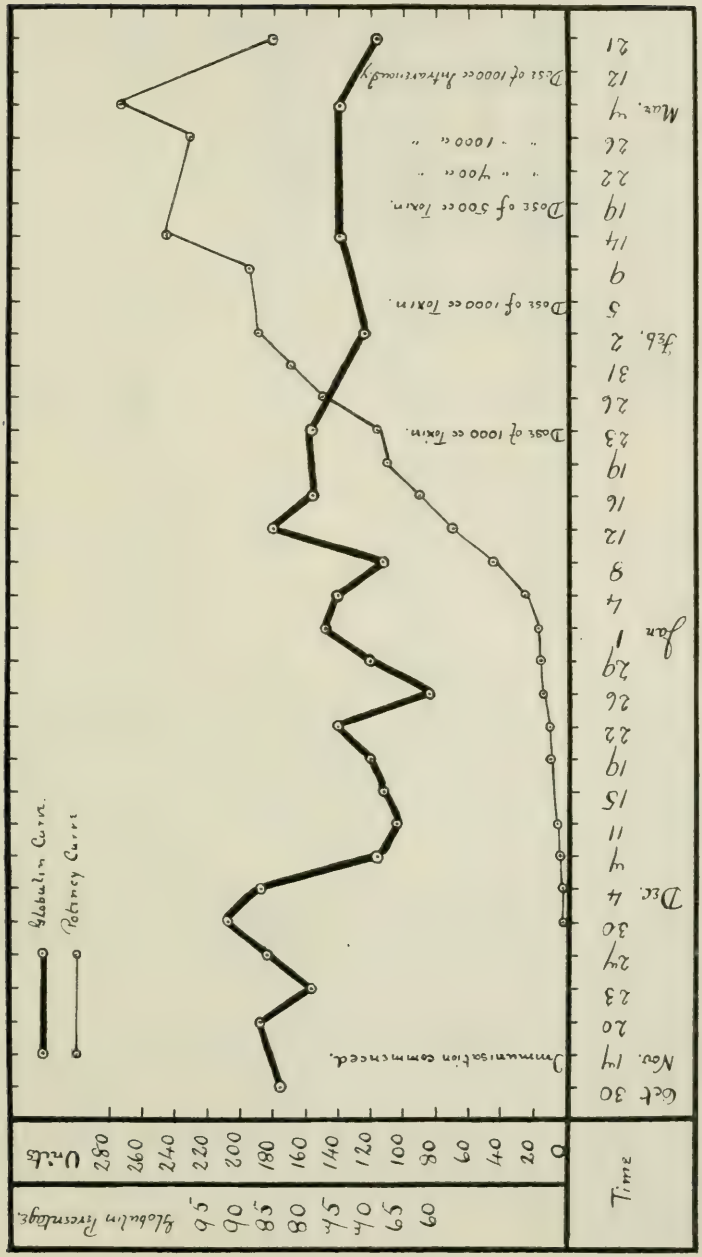

CHART I.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid during immunisation of horse (Plug).

TABLE II.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
Oct. 30 '05	·8532	·7191	84	·1341
Nov. 20	·8816	·7608	87	—
23	·8330	·6618	79	—
27	·8702	·7529	86	—
30	·8034	·7400	92	—
Dec. 4	·8220	·7182	87	—
7	·8987	·6247	69	—
11	·8201	·5945	66	—
15	·8304	·5710	68	—
19	·8006	·5648	70	—
22	·8334	·6278	75	—
26	1·0622	·6532	61	—
29	·9382	·6582	70	—
Jan. 1 '06	·9273	·7213	77	·2060
4	·9372	·7064	75	·1696
8	·8906	·6074	68	—
12	·7460	·6653	89	—
16	·9294	·7390	79	—
23	·9711	·7727	79	·1478
Feb. 2	1·0272	·7357	71	·2370
14	·9561	·7204	75	—
Mar. 7	·9083	·6811	75	·1804
21	·8468	·5890	69	·1728

Horse (Plug).

In the above table (Table II.) are recorded estimations of the total coagulable proteid, and total globulin with a few albumin estimations. In all cases the amounts given are those derived from 10 c.c. serum. Starting with an initial value of ·8532 gm. on Oct. 30 before immunisation, the total proteid exhibits very slight fluctuations throughout the period ending Dec. 22 the average value being ·8406 gm. During the next period ending Jan. 23 the total proteid showed a slight though quite appreciable rise, the average for the period being ·9252 gm. From Jan. 23 onwards this higher level was maintained.

Regarding the globulin variations it will be seen in the first place that the initial value was much higher than that recorded by Hammarsten for the normal horse. In fact the value ·7191 gm. is nearly double the normal value. Thus three normal horses described by Hiss and Atkinson had initial values of ·3085 gm., ·4342 gm., ·3988 gm. in 10 c.c. serum while the horse "Wright," presently to be described, had an initial globulin value of ·4365 gm.

The fluctuations in the percentage globulin-content of the total proteid will be more readily followed from Chart I.

No progressive rise in the globulin-content occurred during the process of immunisation. While the total proteid remained stationary during the period Nov. 17 to Dec. 22 the globulin-content maintained its initial level only for a short time. It then fell persistently attaining its lowest level on Dec. 26.

During the second period when the total proteid was at a higher level, the globulin-content slowly rose to its initial value beyond which it never mounted. The values recorded in the period Jan. 23 to Mar. 21, show that a fall to a lower level had again occurred.

The rise in the total proteid from Dec. 22 onwards was due not to an increase of the globulin but of the albumin fraction.

The important question, whether our failure to obtain a high-grade antitoxic serum in this horse, is in any way related to the peculiar behaviour of the globulin, will be discussed later.

Immunisation of Goat (Mephistopheles).

In the immunisation of the goat, almost exactly the same procedure was adopted as in the case of the horse except that the initial dose of toxin was only ·001 c.c.

The inoculations were made not intramuscularly but subcutaneously in the dorsal region over the erector spinae mass. Before immunisation the normal serum was tested for the presence of normal antitoxin and quantitative estimations of the proteids made. Further in view of the possibility of globulin-variations due to lowered condition etc. the animal was carefully weighed every week. The annexed table (Table III.) shows the scheme of immunisation and the potency of the serum on the different dates.

It was found that the toxin doses were well tolerated up to the middle of February when the animal began to show signs of weakness. After the dose of 100 c.c. the goat developed slight paresis in the hind limbs but appetite remained good and a further injection of 150 c.c. was made on the 19th February. The paresis however became worse and latterly affected the fore limbs so that the animal had to lie on its side. Though thus prostrated appetite remained good but death took place suddenly on 3rd March probably from heart failure.

At the post mortem the stomach was enormously dilated with food-stuffs. Apart from haemorrhagic oedema of the lower pulmonary lobes there were no gross changes in the organs. The serum obtained post mortem had between 20 and 30 units per c.c.

TABLE III.

Date	Toxin-dose	Weight	Potency (in units)
Nov. 6 '05	—	—	$<\frac{1}{5}$
9	—	—	$<\frac{1}{20}$
20	—	—	$\frac{1}{40}$
Dec. 1	·001 c.c.	90 lbs.	—
5	·002	—	—
9	·004	—	—
13	·01	89	—
18	·02	—	—
23	·04	—	$<\frac{1}{3}$
27	·1	80	—
30	·2	—	—
Jan. 3 '06	·5	88	—
8	1	—	—
12	2	87	—
16	3	—	4
19	6	80	—
23	12	—	5—10
29	25	—	—
Feb. 3	50	—	<5
7	50 (new tox.)	—	—
12	100	—	5—10
19	150	—	15—20
Mar. 3	Death	—	20—30

From the potency curve (see Chart II.) it will be seen that it required six weeks' immunisation to raise the antitoxin-content to one unit per c.c. Thereafter progress was more rapid, but for a period of three weeks, Jan. 23 to Feb. 12, the potency appears to have remained more or less stationary with an interval of depression.

After the 12th Feb. the antitoxin-content rose markedly and continued to do so up to the fatal conclusion in spite of the diphtheritic paralysis. Records of immunisation of goats against diphtheria-toxin are not numerous and it does not appear that these animals are capable of producing serum of high potency.

In one goat previously immunised at this Institute a potency of 40 units was reached. The goat serum with which Pick worked had a very low antitoxic value inasmuch as ·1 c.c. was required to neutralize 10 lethal doses of a toxin whose M.L.D. was only about ·01 c.c. (The toxin employed here in the immunisation of the horses and goat had a M.L.D. of ·002 c.c.)

In Table IV. are recorded the values of the total proteid, total globulin and albumin, during the immunisation of the goat. The figures for the normal serum of Nov. 6 show, as in the horse, a marked

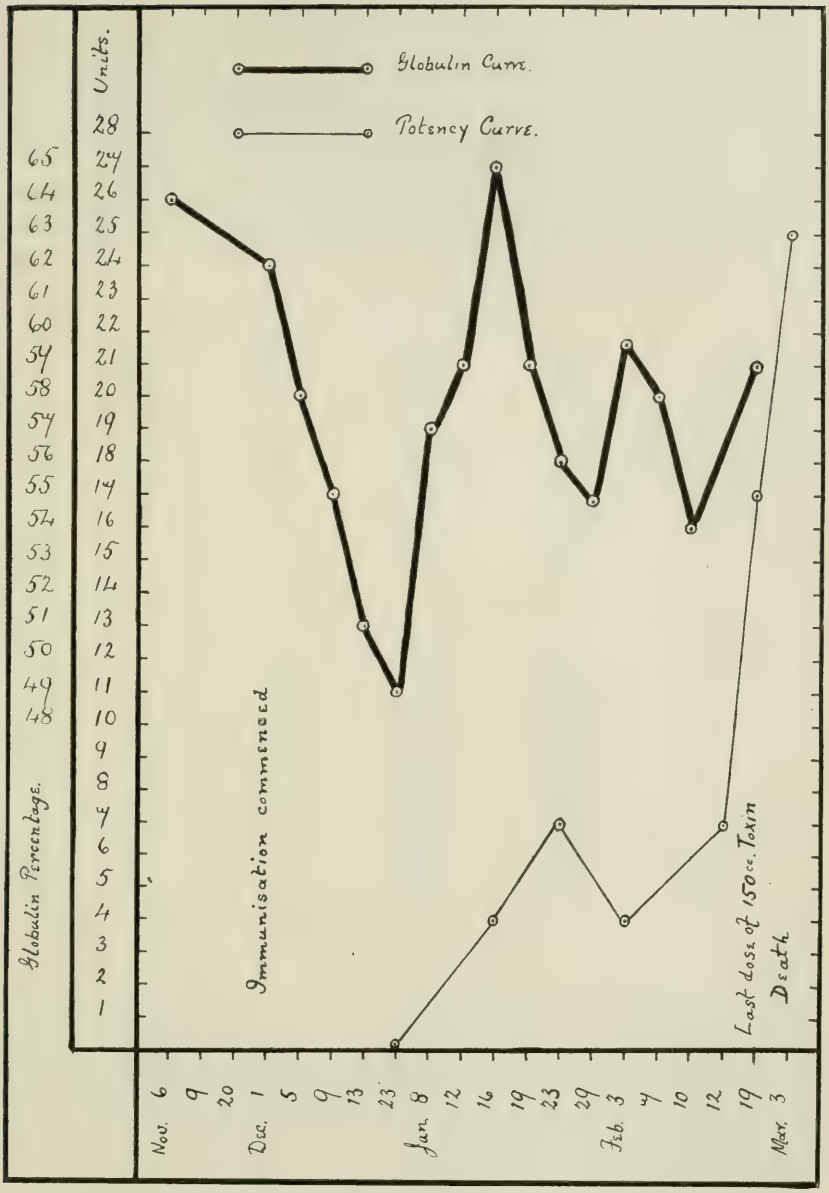

CHART II.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid, during the immunisation of goat (Mephistopheles).

preponderance of the globulin—over the albumin—fraction. The total proteid shows a pronounced and fairly progressive rise from ·5974 gm. on Dec. 1 to ·8555 gm. on Jan. 29. This increase which was equivalent to one of 43 % on the initial value greatly exceeded in amount the corresponding increase in the horse "Plug," but when we consider the tables we find that the very slight increase of total globulin during immunisation, is quite insufficient to account for the greatly increased total proteid. Indeed, as will be apparent from Chart II. the percentage globulin-content of the total proteid fell markedly during the first three

TABLE IV.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
Nov. 6 '05	·5867	·3775	64	·2092
Dec. 1	·5974	·3739	62	—
5	·6668	·3868	58	—
9	·6908	·3820	55	·2261
13	·6161	·3156	51	—
18	·6388	—	—	—
23	·6428	·3204	—	—
27	—	—	—	—
30	—	—	—	—
Jan. 3 '06	—	—	—	—
8	·7430	·4252	57	—
12	·7410	·4399	59	—
16	·7150	·4644	65	—
19	·7608	·4548	59	—
23	·8128	·4554	56	—
29	·8555	·4702	54·9	—
Feb. 3	·7615	·4547	59·7	·2873
7	·7669	·4476	58	—
10	·7581	·4098	54	—
19	·6852	·4047	59	·2597

Goat (Mephistopheles).

weeks. It then rose gradually till it attained a value slightly above the initial figure of 64 %. The rise, however, was only temporary and was succeeded by a second fall which lasted with slight fluctuations during the period of maximum potency. It must be said, therefore, that the great increase in the total proteid was due in far greater measure to the albumin than to the globulin fraction

Such a result, in view of the fact that the globulin is the antitoxin carrier, seems rather surprising and will demand our consideration later.

Immunisation of Horse (Wright).

As the horse "Plug" proved unsatisfactory for the production of high grade antitoxin and consequently did not permit of our drawing any very definite conclusions regarding the relationship of the serum-globulins to the potency, it was decided to immunise another horse.

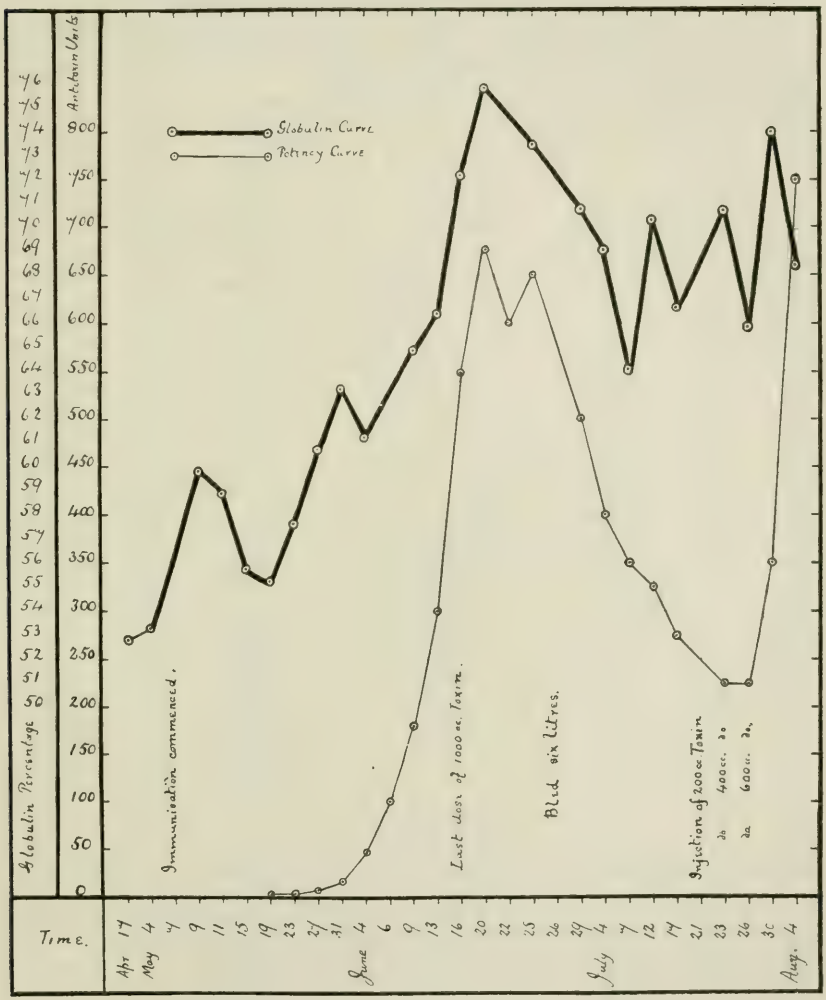

CHART III.

Chart showing the course of antitoxin-development and the variations in the percentage globulin-content of the total proteid during the immunisation of horse (Wright).

The procedure adopted was in all respects similar to that in the case of "Plug" except that the inoculations were made, where possible every second day. No delay was experienced until doses of 200, 400, and 600 c.c. were administered when the inoculations were postponed for a day or two to allow local swelling to subside.

In the following table (Table V.) are recorded the toxin-doses and potency of the serum during the course of immunisation.

It will be observed that "Wright" reacted in a much more characteristic way to diphtheria-toxin than "Plug," and the potency curve (see Chart III.) may be considered a fairly typical one.

TABLE V.

Date	Toxin	Potency (in units)
April 17 '06	—	$<\frac{1}{4}$
May 7	·01	—
9	·02	—
11	·04	$<\frac{1}{8}$
13	·1	—
15	·2	—
17	·5	—
19	1	2—4
21	2	—
23	4	2—4
25	8	
27	15	8
29	30	—
31	50	16
June 2	100	—
4	200	>40
6	400	100
9	600	180
13	—	300
16	1000	550
20	—	675
22	—	600
25	—	650
26	Bled 6 litres	—
29	—	500
July 4	—	400
7	—	350
12	—	325
17	—	250—300
21	200	—
23	400	225
26	600	200—250
30	—	>300
Aug. 4	Bled 4 litres	750

After the first bleeding, the potency gradually fell to 300 units and under, but a second series of inoculations caused it to mount upwards again to a level higher than that attained at the end of the first series. Such a phenomenon is not unfrequent.

In Table VI. are recorded the values of the total proteid, total globulin and albumin during immunisation. A series of euglobulin and pseudoglobulin estimation are also entered.

TABLE VI.

Date	Total proteid	Total globulin	Albumin	Euglobulin	Pseudo-globulin	Globulin percentage	Albumin percentage
Apr. 17 '06	·8266	·4365	—	—	—	52·8	—
May 4	·7954	·4241	—	—	—	53·3	—
9	·8440	·5050	·2734	·1600	·3333	59·8	32·3
11	·7964	·4692	—	—	—	58·9	—
15	·8289	·4622	·2636	·0952	·3544	55·7	31·8
19	·7278	·4021	·2826	—	—	55·2	38·8
23	·7884	·4546	·2545	—	—	57·6	32·2
27	·8226	·4998	·3027	·0916	·3893	60·7	36·7
31	·8043	·5094	·3005	·1495	·3131	63·3	37·3
June 4	·8065	·4941	·3046	·1358	·3072	61·2	37·7
9	·8346	·5419	·2456	—	—	64·9	29·4
13	·9452	·6278	·1930	·2178	·4295	66·4	20·4
16	1·0234	·7379	·2363	·1146	·6040	72·1	23·0
20	1·0600	·8046	·2004	·2934	·4080	75·9	18·9
25	·9387	·6893	·2043	·2795	—	73·4	21·7
29	·8519	·6026	·1916	·1320	·4286	70·7	22·5
July 4	·8190	·5655	·2151	—	—	69·0	26·2
7	·7963	·5102	·2333	—	—	64·0	29·3
12	·8225	·5785	·2596	—	—	70·3	31·5
17	·7853	·5238	·2874	—	—	66·7	36·6
23	·8398	·5940	—	—	—	70·7	—
26	·9342	·6159	—	—	—	65·9	—
30	·9429	·6986	—	—	—	74·0	—
Aug. 4	·9276	·6343	—	—	—	68·3	—

Horse (Wright).

During the first month the total proteid remains fairly constant with slight fluctuations, but a rapid rise takes place between the 9th and 20th June, a period corresponding to the maximum antitoxin development. This rise is succeeded by an appreciable fall during the resting period following the bleeding. A second rise, however, makes its appearance after the second series of inoculations. Roughly the total proteid rose from 8 % before immunisation to 10 % at the period of maximum antitoxin development.

Apart from slight fluctuations, the absolute amount of globulin rises very slowly during the first three weeks so that the percentage globulin-content of the total proteid also rises (see Chart III.). The increase is much more rapid in the second month when the potency is rising the figure for 20 June being almost double the initial figure before immunisation. At the period of maximum globulin-content the albumin percentage is reduced to 18·9 %.

We have, therefore, to record in this horse a very marked rise in the globulin-content coincident with the development of a high potency and with a reduction of the albumin fraction. Regarding the euglobulin and pseudoglobulin estimations, the results are somewhat indefinite but would at least indicate that in the globulin increase, both fractions are implicated and the euglobulin more so than the pseudoglobulin. The result is in more or less agreement with that recorded by Joachim and to which we have already referred.

After the bleeding and during the resting stage the total proteid and total globulin fall while the albumin remains more or less stationary or even tends to rise. When the inoculations were renewed, the total proteid and total globulin again rose but more slowly and even at the period when the potency had reached its second maximum the total globulin had not quite reached a value equal to its previous maximum at the end of the first series of inoculations. The globulin values, however, never attain the low limits prevailing before the commencement of immunisation and during the earlier part of the process.

The question of the influence of inanition or repeated bleedings, on the constitution of the blood plasma has undoubtedly to be considered in all experimental work designed to bring out a causal connexion between rise in potency and rise in the globulin fraction of the blood serum. In this connexion we may cite the work of Githens (1904) who found that in hungering dogs a rise in the globulin fraction occurred especially affecting the fibrinoglobulin and euglobulin, while after repeated bleedings the albumin-content rose markedly.

This peculiar relationship of the albumin and globulin fractions inclined the author to believe that the albumin behaves as if it came nearest to the "Nahrungseiweiss" and suggested the possibility that the globulins under these conditions might be derived from the albumins either inside or outside the circulating blood.

Glässner (1905) investigated the changes in the blood plasma before and after the immunisation of rabbits with bacterial emulsions, bacterial

toxins and proteid bodies as horse or ox sera. He came to the conclusion that a rise in the globulin fraction was only marked in those animals which had lost weight during immunisation, and that consequently a rise in the globulin fraction could not be considered an essential concomitant of the formation of antibodies but only as a secondary symptom depending on inanition. Provided immunisation is cautiously proceeded with and the animals not allowed to get into an enfeebled condition, a rise in globulin need not necessarily follow. Glässner thinks his views are supported by the results obtained by Githens already quoted but as has also been pointed out recently by Moll (1906) the globulin values obtained by Githens after inanition are not to be compared to the high values obtained by immunisation.

In Moll's own experiments on fasting dogs and rabbits no marked rise in the globulin fraction was noted. In two cases the globulin even fell. By immunising with horse serum however a great rise in globulin was observed and was accompanied by the development of marked precipitin formation. He does not believe that the rise in globulin is an inanition effect though it is possible that pure inanition may influence this phenomenon during immunisation.

Before commencing our work we had in view the possibility of inanition or loss of weight as possible disturbing factors. Unfortunately the horses were not weighed during immunisation but it is the general experience in this Institute that, so far from losing weight the horses appear actually to put on flesh. They receive as much nourishment as they will take and enjoy regular exercise. It is questionable, however, how far we are to consider this apparent well-being as a criterion of an undisturbed metabolism. The occasional occurrence of waxy disease, hepatic haemorrhage etc. causing sudden death in apparently healthy horses undergoing immunisation for long periods, suggests that metabolism may be profoundly altered without much visible sign being afforded of its effects.

Determinations of the antitoxin-contents of the proteid fractions in the horse and goat.

We have already referred to Pick's work showing that in the horse the pseudoglobulin is the antitoxin carrier, while in the goat the euglobulin performs that function. As no confirmatory evidence on this important question is yet available, and as the possibility was always present that the sera of different horses might comport themselves

differently in regard to the antitoxin-contents of their respective fractions, it seemed desirable to test the potency of a number of proteid solutions isolated from horse and goat serum at different stages in immunisation.

The separations were all performed by the ammonium sulphate method and the various precipitates were dissolved in distilled water. Where the final fluid exceeded in bulk that of the original serum quantity taken, the necessary allowance was made in testing the potency.

For each test three or four guinea-pigs of standard weight were employed.

Experiments on Horse serum.

Exp. I. Total globulin.

Serum employed: Plug's serum of March 7, 1906 containing 250—300 units per c.c.

Mode of separation: A single precipitation with $(NH_4)_2SO_4$ to half saturation.

Dissolved precipitate tested for 100, 200 and 250 units in a series of three pigs.

Result: Pig No. (3) died on 4th day while others lived.

Remarks: The globulin contained practically all the antitoxin.

Exp. II. Total albumin.

Mode of separation: Filtrate from Exp. I.

Tested for 10, 20 and 40 units in three pigs.

Result: All three pigs died on 2nd day.

Remarks: Apparently the albumin contains none of the antitoxin.

Exp. III. Euglobulin.

Serum employed: Plug's serum of March 21, 1906 containing 200 units per c.c.

Mode of separation: A single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 10, 20 and 40 units in three pigs.

Result: All three pigs lived.

Remarks: No conclusion can be drawn as a certain amount of the pseudoglobulin may have been mechanically carried down in the first precipitate.

Exp. IV. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. III.

Tested for 50, 100 and 150 units.

Result: Pig No. (1) lived, pig No. (2) died on 4th day, and pig No. (3) died on 2nd day.

Remarks: The combined pseudoglobulin and albumin contained more than half the antitoxin after a single separation from euglobulin by one-third saturation.

Exp. V. Euglobulin.

Same serum as in Exp. III.

Mode of separation: Complete separation of euglobulin by repeated precipitation with $(NH_4)_2SO_4$.

Tested for 10, 20 and 40 units.

Result: Pig No. (3) died on 2nd day while the others lived.

Remarks: The euglobulin fraction even after complete separation from the pseudoglobulin and albumin contained 20 units per c.c.

Exp. VI. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. V.

Tested for 50, 100 and 150 units.

Result: Pig No. (3) died on 3rd day while others lived.

Remarks: The pseudoglobulin-albumin therefore contained three-fourths of the antitoxin.

Exp. VII. Euglobulin.

Serum employed: Serum of "Togo" containing 600 units per c.c.

Mode of separation: Single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 25, 50 and 100 units.

Result: All three pigs lived.

Remarks: The remarks on Exp. III. apply here also.

Exp. VIII. Euglobulin.

Same serum as in Exp. VII.

Mode of separation: Complete separation from pseudoglobulin by repeated precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 25, 50 and 100 units.

Result: All three pigs died on 2nd day.

Remarks: The euglobulin apparently contains none of the antitoxin.

Exp. IX. Pseudoglobulin-albumin.

Mode of separation: Combined filtrates from Exp. VIII.

Tested for 400, 500 and 600 units.

Result: Pig No. (1) lived while pig (2) and (3) died on 2nd day.

Remarks: The pseudoglobulin-albumin certainly contained over two-thirds of the antitoxin but some loss of antitoxin has apparently occurred in the preparation of the different fractions.

Experiments on Goat serum.

Exp. I. Total globulin.

Serum employed: Mephistopheles' serum of 19 Feb. 1906 containing 15—20 units per c.c.

Mode of separation: Complete separation from albumin by repeated precipitation with $(NH_4)_2SO_4$ to half saturation.

Tested for 5, 10 and 15 units.

Result: Pig No. (3) died on 3rd day while the others lived.

Remarks: The globulin appears to contain all the antitoxin.

Exp. II. Euglobulin.

Serum employed: Meph. serum of Feb. 7, 1906 containing about 5 units per c.c.

Mode of separation: Single precipitation with $(NH_4)_2SO_4$ to one-third saturation.

Tested for 3, 4 and 5 units.

Result: Pig No. (3) died on 3rd day while the others lived.

Remarks: The euglobulin appears to contain all the antitoxin but the experiment is not conclusive, as some of the pseudoglobulin may have been carried down in the first precipitation.

Exp. III. Pseudoglobulin-albumin.

Mode of separation: Filtrate from Exp. II.

Tested for 2, 3 and 4 units.

Result: All three pigs died on 2nd day.

Remarks: Evidently a single precipitation with $(NH_4)_2SO_4$ to one-third saturation removed all the antitoxin, leaving none in the pseudoglobulin-albumin filtrate.

Exp. IV. Euglobulin.

Serum employed: Meph. serum of Jan. 29, 1906 containing about 5 units per c.c.

Mode of separation: Complete separation from pseudoglobulin-albumin by repeated one-third saturation with $(NH_4)_2SO_4$.

Tested for 2, 3, 4 and 5 units.

Result: All pigs died on 2nd day.

Remarks: It would appear that on this occasion none of the antitoxin was contained in the euglobulin fraction.

Exp. V. Euglobulin.

Serum employed: Meph. serum of 19 Feb. 1906, containing 15—20 units per c.c. (same serum as in Exp. I.).

Mode of separation: Complete separation from pseudoglobulin-albumin by repeated one-third saturation with $(NH_4)_2SO_4$.

Tested for 5, 10 and 15 units.

Result: All three pigs died on 1st day from toxaemia.

Remarks: Taken in conjunction with Exp. I. it would seem that on this occasion all the antitoxin must have been contained in the pseudoglobulin fraction.

General Summary of Experimental results.

There appears to be no doubt that the greater part, if not the whole of the antitoxin of horse serum is contained in the pseudoglobulin fraction. In the horse "Togo" which was yielding serum of high potency not more than 2—3 % of the antitoxin could have been present in the euglobulin when completely separated from the pseudoglobulin.

In the horse "Plug," however, the euglobulin when completely separated from the pseudoglobulin still contained fully 10 % of the antitoxin. It will be remembered that from Feb. 14, 1906 onwards

little change took place in the potency in spite of repeated stimulation. The serum of Mar. 21 (see Exp. V.) was drawn at a time when the potency was evidently falling or at least stationary so that the conclusion is forced upon us that in the serum of a refractory horse which responds sluggishly or not at all to stimulation there may be no such sharp delimitation of antitoxin to the pseudoglobulin as in the serum horses more susceptible to stimulation.

In order to obtain reliable data on this point, however, it would be necessary to make a larger series of experiments on the above lines, at different stages in the immunisation of refractory horses.

In the case of the goat I am unable to confirm Pick's statement that the antitoxin is invariably linked on to the euglobulin fraction. The analysis of sera on 29th Jan. and 19th Feb. showed that the euglobulin contained none of the antitoxin, while on the intervening date, 7th Feb., the euglobulin (though here incompletely separated) contained all the antitoxin. It is interesting to observe that between the 23rd Jan. and 12th Feb. the potency remained stationary with an intervening depression so that again we have the possibility suggested to us that during a refractory period which corresponds in all probability to an abnormal metabolic activity, the distribution of the antitoxin-molecules may be altered.

The fact too that in the globulin-increase during immunisation the euglobulin appears to take a larger share than the pseudoglobulin which acts as the antitoxin-carrier seems to show that some compensatory mechanism is at work. In the horse whose reaction to stimulation is prompt and is evidenced by increased potency, we may presume that a more or less constant number of pseudoglobulin molecules has to combine with or hold together a very largely increased number of antitoxin molecules. In order to prevent any diversion of these pseudoglobulin molecules to simple functions of nutrition the euglobulin undergoes what one may call a "compensatory hypertrophy."

In the refractory animal, however, or during a period of stationary potency such a mechanism may not exist, or once established may readily be upset in which case a redistribution of the antitoxin molecules would not be an unexpected phenomenon. Hitherto no explanation has been afforded of the different reactions displayed by different horses to diphtheria-toxin. From the data furnished by the immunisation of "Plug" and "Wright" there would seem to some intimate relation between the amount of antitoxin developed and the quantity and quality of the globulins. In both cases (as also in the goat whose power

of elaborating antitoxin is very limited) the total proteid was increased, but in the first case the increase affected largely the albumin, and in the second case, the globulin solely. In the goat the albumin was mostly implicated in the increase of total proteid. Now both horses had practically the same amount of total proteid initially but in the case of "Plug" the globulin fraction preponderated enormously over the albumin fraction. It was only at the height of antitoxin-production that the globulin of "Wright" attained a value equal to that of "Plug" initially. Had the globulin fraction of "Plug's" serum increased in the same proportion as that of "Wright," quite apart from increase of potency, the total proteid would have consisted practically entirely of globulin.

But there is no doubt a physiological limit to the amount of globulin in serum and that limit was attained in "Plug," for some unexplained reason, before immunisation began. Hence the increase in the total proteid fell largely on the albumin fraction which however does not functionate as an antitoxin carrier. If in view of Hiss and Atkinson's work and the data supplied by "Wright" we are to consider increase of globulin as a necessary concomitant of high grade antitoxin-development then the failure of "Plug" to yield such high grade serum is not surprising.

We use the term "high grade serum" advisedly because there is no doubt that a certain degree of potency is attainable without any very marked disturbance in the constitution of the serum-proteids. In this connexion it seemed advisable to exclude any possible globulin variation that might occur from the injections of large quantities of bouillon alone, apart from the toxin they contain. To make certain on this point a horse "Lister" was inoculated with increasing quantities of alkaline bouillon according to the following scheme.

The bouillon employed was the same as that used in the production of diphtheria-toxin. It was kept in the incubator for ten days and finally filtered through a Berkefeld in order to approximate as closely as possible the conditions of the toxin-containing bouillon.

The initial dose which was inoculated intramuscularly was 50 c.c. Thereafter doses of 100, 200, 400, and 800 were given at intervals of two or three days.

After each injection a well marked pyrexia was evident but otherwise no untoward symptoms appeared.

Before the commencement of the inoculations estimations were made of the total proteid, total globulin and albumin.

In Table VII. are recorded the various proteid estimations throughout

the period of inoculation. It will be seen that no appreciable change took place in the amount of total proteid. The globulin fluctuations were also very slight as will also be evident from Chart IV. which shows the fluctuations in the percentage globulin-content of the total proteid.

TABLE VII.

Date	Total proteid	Total globulin	Globulin percentage	Albumin
July 31 '06	·7493	·4880	65·1	·2702
Aug. 8	·7898	·4654	58·9	—
13	·8063	·5246	65	·3201
16	·8478	·5006	59	·2074
20	·7590	·4639	61·1	·2070

Horse (Lister).

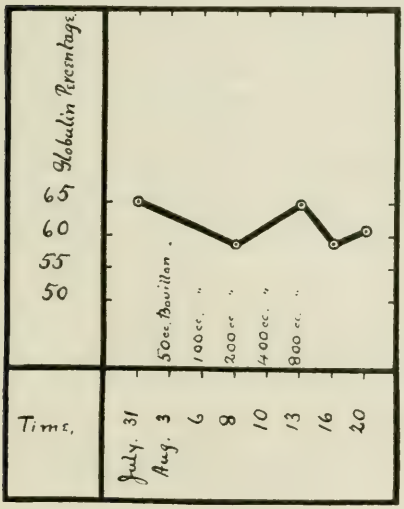

CHART IV.

Chart showing the variations in the globulin-content of the total proteid during the course of the Bouillon-injections (Lister).

We may therefore exclude the inoculation of large quantities of bouillon as a possible factor in the production of the marked globulin-increase which occurs during the immunisation of susceptible horses with diphtheria-toxin.

Summary and Conclusions.

1. During the immunisation of a horse which ultimately failed to yield high grade antitoxic serum, the globulin-content of the total proteid showed no tendency to increase. The slight rise in total proteid which occurred was due to an increase in the albumin fraction. It is probable that the failure of this horse to yield high grade antitoxin was in some way connected with the initial high globulin-content of the serum.

2. During the immunisation of a goat, the rise in total proteid affected mainly the albumin fraction and the globulin fraction in lesser degree.

3. During the immunisation of a horse which ultimately yielded high grade antitoxic serum, the percentage globulin-content of the total proteid, progressively increased. This increase affected the euglobulin fraction more than the pseudoglobulin fraction.

4. In the horse the pseudoglobulin contains the greater part if not all the antitoxin but it seems probable that this relationship holds good only when the antitoxin-content of the serum is steadily rising.

5. In the goat the antitoxin-content of the euglobulin and pseudoglobulin fractions may vary at different periods in the course of immunisation.

In conclusion I have to express my thanks to Dr Dean the Bacteriologist-in-charge for kindly help and suggestions in the course of this work.

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ADDENDUM.

In the *Proceedings of the Society for Experimental Biology and Medicine*, vol. IV, No. 1, New York, Dec. 1, 1906, is contained the abstract of a paper by Gibson and Collins on the fractionation of agglutinins and antitoxin. These authors report:—"Precipitation of anti-diphtheria goat serum showed that about half the antitoxin remained in the pseudoglobulin; practically none was found in the euglobulin while the $\frac{1}{3}$rd saturated $(NH_4)_2SO_4$ solution washings contained the balance." The results of their experiments, so far as they had gone, appeared to indicate the unreliability of Pick's differentiation of the antibodies by fractional precipitation of the globulins.

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NOTES ON THE LEUCOCYTE-REACTION DURING THE IMMUNISATION OF THE HORSE AND GOAT WITH DIPHTHERIA TOXIN.

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In the foregoing paper details have been given of the immunisation with diphtheria toxin of three animals (two horses and one goat). In the case of the horse "Plug" and goat "Mephistopheles" the opportunity was taken of investigating the question of the leucocyte reaction with a view to determine whether any notable changes in the leucocytic formula took place as the result of the frequent injections of toxin and the increased elaboration of antitoxin. The few investigations which have been recorded on the blood-changes during diphtheria immunisation have unfortunately led to somewhat contradictory conclusions.

Nicolas and Courmont (1897) were the first to study this question in any detail. These authors recorded the variations in the total leucocytes during the immunisation of several horses. Immunisation was begun with iodised toxin and continued every second or third day with gradually increasing doses of pure toxin. No fixed rule was followed however. The final dose after about fifty injections was only 60 c.c. toxin. Consequently the intermediate doses of 5 c.c., 10 c.c., 15 c.c., 20 c.c., 30 c.c., 40 c.c., and 50 c.c., were many times repeated. Counts made at rather irregular intervals during the course of immunisation did not show any reaction in the direction of hyperleucocytosis. The average leucocyte count during immunisation varied only very slightly from that previous to the commencement of the inoculations. On two or three occasions, counts were made every two or three hours, after the injection, but here again no notable reaction was evident. Nicolas and Courmont concluded that no conspicuous leucocyte-reaction was to be

detected either at the beginning or at an advanced stage of the injection period and that therefore immunity could be developed without any essential leucocyte change. If hyperleucocytosis results at all it is merely a sign of grave intoxication and indicates that the toxin dose has been too strong.

The work of these authors was severely criticised by Besredka (1898) who laid stress on the necessity of paying attention principally to the behaviour of the polynuclear cells and not of the absolute leucocyte count. Further, while pointing out that Nicolas and Courmont had failed to give any account of the antitoxin yield of their horses, Besredka affirmed that he would not have been surprised at the absence of leucocyte-reaction in the absence of any great development of immunity. It seems doubtful how far this latter criticism of Besredka is justifiable.

This author immunised a goat with diphtheria toxin and showed that each dose of toxin was followed by a conspicuous leucocyte reaction in which the polynuclear cells were mainly implicated. During the later stages, however, there was some evidence that the polynuclear cells did not react so promptly and that the mononuclear cells participated more largely in the reaction.

It may be noted that immunisation was begun with heated toxin and followed up with the unheated fluid. The final dose, after about three months' immunisation, was only 1·5 c.c. of a toxin whose lethal dose for a guinea-pig of 500 gm. was $\frac{1}{50}$ c.c. At the end of this period he was able to demonstrate that ·5 c.c. of the goat serum when mixed with 20 fatal doses of toxin (= ·20 c.c.) protected a guinea-pig of 500 gm. weight. No mention of his method of taking blood-samples from the goat is made by Besredka.

In reply to the latter's criticism Nicolas and Courmont (1898) maintained that their horses had undoubtedly developed antitoxin as a result of the injections. Further, they refer to the temporary paraplegia of Besredka's goat as being due to incautious inoculation. Under such conditions, they assert, a certain degree of hyperleucocytosis would not be an unexpected phenomenon.

A second contribution to the subject was made by Nicolas, Courmont and Prat (1900). Three animals were immunised, a horse, a goat, and a donkey. The polynuclear cells were estimated in addition to the absolute counts. Each animal received increasing doses of toxin up to 17 c.c., which was the final dose. In the goat the initial dose was $\frac{1}{500}$ c.c. of iodised toxin. The counts were made every two or three days, but no hyperleucocytosis was ever demonstrable. Indeed, it

appeared that a hyperleucocytosis associated with a hypopolynucleosis was most in evidence during the immunisation. In summing up their results these authors adhere to their previous statements that immunisation is possible without any essential leucocyte reaction. Butjagin (1902) without giving numerical details, agrees with Nicolas and Courmont, that the elaboration of antitoxin need not be associated with any notable leucocyte reaction.

The leucocyte reaction in the goat.

In the case of "Mephistopheles" (as also of "Plug") the samples were always taken from the jugular vein by cannula, as it was found that samples taken from the ear gave fallacious results. The skin of the goat's ear is exceedingly tough and elastic, so that after puncture of the vein the blood did not flow freely but tended to gather in the subcutaneous tissue, forming a lump which was slowly absorbed. In Table I. are recorded, in relation to the toxin doses the total leucocyte counts and the percentages of the various leucocyte forms during the immunisation of the goat.

From Dec. 1 to Feb. 7 the counts were made every four days, corresponding with the toxin-injections. After the large doses inoculated on Feb. 7 and 12, counts were made every day, in order to determine more accurately the course of the leucocytes in response to massive doses. Also, on several occasions prior to the commencement of immunisation, the leucocytic formula was determined, the percentage of the non-granular cells being about 10 % higher than that of the polynuclear cells. On the first day of the injections the relation of the non-granular cells to the polynuclear cells was as 55·2 : 43·6.

Let us consider the period from Dec. 1 to Jan. 16. It will be seen from the table that the effect of one injection had generally disappeared before the next came on, with notable exceptions on Dec. 9, Dec. 30 and Jan. 16. On these latter occasions a marked relative polynuclear leucocytosis still remained after the preceding injection. Also on Dec. 30 and Jan. 16 the absolute leucocyte counts were comparatively high.

In the period from Jan. 19 to Feb. 7 the polynuclear cells predominated markedly on the fourth day after each injection. The daily counts after the injection of 50 c.c. toxin on Feb. 7 and of 100 c.c. toxin on Feb. 12, show clearly that the slight resultant hyperleucocytosis is mainly due to an increase in the polynuclear cells while the mononuclears show only slight fluctuations.

TABLE I. Goat (Mephistopheles).

Date	Toxin	Total leucocytes	Small lymphs.	Large lymphs.	L. monos. and trans.	Non-gran. percentage	Polymorphos.	Eosinophils	Mast-cells
Dec. 1	·001	9,350	51·6	2	1·6	55·2	43·6	·8	·4
5	·002	9,650	48	6·1	3·5	57·6	40	1·7	·03
9	·004	9,200	40·6	2·8	1·8	45·2	53·2	1	·6
13	·01	8,300	38	9·5	6·2	53·7	43·7	1·7	·7
18	·02	6,650	51·8	4·4	1	57·2	41·2	1·2	·4
23	·04	8,350	71·1	1·1	·9	73·1	24·3	2·4	0
27	·1	7,900	48·8	2·9	·1	51·8	45·6	1·8	·6
30	·2	11,000	25·3	3·4	·5	29·2	65	4·8	·9
Jan. 3	·5	7,700	49·8	4·4	1·7	55·9	42·3	·8	·9
8	1	6,850	55·3	8·6	·1	64	33·3	2·6	·1
12	2	6,400	41·9	7·9	2·6	52·4	44·2	2·6	·7
16	3	10,500	23·6	4·6	2·8	31	67·9	·8	·2
19	6	9,500	41·5	5·2	·2	46·9	52	·9	0
23	12	8,300	38·8	1·2	0	40	56·6	2·6	·7
29	25	5,800	39·9	7	1·9	48·8	47·5	3·3	·2
Feb. 3	50	8,000	38·2	1·1	·4	39·7	58·4	1·2	·5
7	50	7,350	45·1	1·8	·8	47·7	50·8	1·1	·4
8	—	7,750	32·3	5·3	1	38·6	60·7	·2	·4
9	—	11,400	36·3	1·5	·9	38·7	60	·6	·6
10	—	7,500	29·3	1·1	1·4	31·8	67·4	·3	·3
12	100	6,400	39	3·4	·9	43·3	55	1·2	·2
13	—	10,600	30·4	·9	·6	31·9	67·6	·3	0
14	—	12,000	30·8	1·4	1·9	34·1	65	·7	0
15	—	8,400	38·4	2·1	·9	41·4	57·3	·9	·3
16	—	9,000	51·7	2·6	0	54·3	43·8	·9	1
19	150	11,400	30·5	·3	0	30·8	68·6	·5	0
20	—	16,000	14·9	·4	0	15·3	84·6	0	0

The absolute values of the non-granular and polynuclear cells after Feb. 7 and 12 are recorded below.

Date	Non-granular	Polynuclear	
Feb. 7	3431	3650	Inoculation of 50 c.c.
8	2926	4620	
9	4332	6840	
10	2325	5025	
11	—	—	
12	2752	3520	Inoculation of 100 c.c.
13	3286	4982	
14	4080	7800	
15	3444	4788	
16	4860	3870	
17	—	—	
18	—	—	
19	3420	7752	Inoculation of 150 c.c.
20	2400	13440	

On the day following the inoculation of 150 c.c. a very marked hyperleucocytosis was present and the polynuclear percentage had mounted as high as 84·6.

Regarding the behaviour of the eosinophile cells and mast-cells no very definite conclusions can be drawn owing to the small numbers of these cells present in the blood of the goat. It would seem, however, that after the injections on Feb. 7, 12, and 19, the eosinophile percentage was considerably reduced.

In Table II. are recorded the leucocyte estimations made during the immunisation of "Plug." Before the commencement of the injections the normal leucocytic formula was ascertained. In marked contrast to the goat, the polynuclear percentage slightly exceeds the non-granular percentage. The leucocytic formula of the horse is also remarkable on account of the high percentage of eosinophile cells.

Let us consider the period from Nov. 17 to Jan. 8.

Although a slight rise in the total leucocyte count was evident on the third day after each of the earlier injections, the percentages of the non-granular and polymorphonuclear cells had invariably returned to their normal values.

On Dec. 4, 15, and 19, however, a marked polynucleosis remained. Also on the 19th the total count remained at the high value of 18,000. It is interesting that the further injection of 8 c.c. toxin on this date did not produce any cumulative effect on the leucocyte reaction as, on the following day, the total count had fallen and the relative polynucleosis had diminished.

TABLE II. Horse (Plug).

Date	Toxin	Total leucocytes	Small lymphs.	Large lymphs.	L. monos. and trans.	Non-gran. percentage	Polymorphos.	Eosinophils	Mast-cells
Nov. 17	·01	8,200	40	2	2·7	44·7	49·4	4·7	1
20	·02	11,650	43·8	1	1·5	46·3	47·1	4·8	1·6
23	·04	12,550	38·8	3·6	3·6	46	48·4	4·4	·9
27	·1	10,166	39·2	3·6	4·2	46	46·3	5·8	·8
30	·2	11,800	42	3·2	3·2	48·4	46·4	4	1
Dec. 4	·5	9,300	36·2	3·3	2	41·5	52·3	4·4	1·7
7	1	10,100	38·5	3·7	1·8	44	49	5·3	1·4
11	2	7,850	45·6	2·1	1·0	48·7	47·6	2·6	·8
15	4	10,050	32·1	5·2	4·5	41·8	53·1	3·8	1
19	8	18,000	15·6	1·6	4·9	22·1	75·5	1·2	1
20	—	12,700	29·6	4·1	3·0	36·7	57·8	4·2	·9
22	15	8,650	46·5	1·8	·8	49·1	47·1	3·1	·5
26	30	10,100	52·6	2·1	·3	55	43	1·1	·6
29	60	10,000	35·8	3·6	3·2	42·6	55	1·8	·7
Jan. 1	120	8,600	33·9	2·0	1·1	37	60	2	·9
4	250	10,000	34·9	3·4	2·4	40·7	55·4	2·2	1·5
8	500	8,300	35·9	1·9	·3	38·1	58·1	2·7	1
9	—	13,750	19·2	·4	0	19·6	79·2	1·1	0
10	—	19,250	17·6	1·9	2·1	21·6	74·8	1·9	1·6
11	—	14,250	26·6	4·2	·7	31·5	65·3	2·6	·4
12	500	8,150	39	2·2	·1	41·3	56·5	1·9	·1
16	800	11,650	36	4	·5	40·5	55·1	3·7	·5
17	—	11,100	15	1·3	·8	17·1	79·4	2·9	·4
18	—	15,800	21·8	2·8	1·7	26·3	70·1	3	·4
19	—	12,800	18·2	2·8	1·6	22·6	74·5	2·4	·3
23	1000	8,400	31·6	3·7	1·8	37·1	59·2	2·7	·9
24	—	19,200	9·9	1·8	·3	12	87·3	·1	·6
25	—	17,100	13·5	·7	3·4	17·6	80·2	1·9	·1
26	—	13,600	28·6	·9	·3	29·8	66·4	2·8	·8
27	—	8,400	42·8	2·5	2·1	47·4	48·1	3·9	·4
29	—	9,250	33·8	2	1·2	37	57·5	5·3	·1
30	—	12,750	30·7	1·2	2	33·9	61	4·3	·7
31	—	8,600	26·9	3	3·3	33·2	62·2	3·9	·5
Feb. 1	—	10,500	33	2·3	1·6	36·9	58·5	3·8	·5
2	—	14,000	31·5	3·8	2·7	38	55·1	5·6	1·1
5	1000	7,150	42·5	3·4	2·1	48	47·6	3·8	·5
6	—	15,500	18·3	·6	·6	19·5	78·6	1·1	·7
7	—	20,300	23·7	1·2	·9	25·8	69·3	3·6	1·1
8	—	12,250	32·5	1·2	1·2	34·9	59·5	3·7	1·8
9	—	8,700	42·4	·4	·9	43·7	47·6	5·7	2·7
10	—	8,500	43	1·8	1·3	46·1	48·9	3·8	1·1

Polynucleosis was also marked on Dec. 29, Jan. 1, 4, and 8. After the injections of 500 c.c., 800 c.c., and 1000 c.c. (*bis*), the blood was examined every day as was done in the case of massive injections in the goat. It will be seen that each of these injections was followed by an enormous rise in the polynuclear percentage although the hyperleucocytosis never exceeded 20,000.

Below are appended the absolute values of the non-granular and polynuclear cells on the days following these massive injections.

After the intravenous injection of 1000 c.c. toxin on March 12, the blood was also examined and it was found that on the day following, the polynuclear percentage had risen to the enormous value of 91·1 while the mononuclear percentage fell to 8·9. On the 14th of March, the polynuclear percentage fell to 83·9 while the mononuclear rose to 15·1.

Date	Non-granulars	Polynuclears	Toxin
Jan. 8	3162	4822	500 c.c.
9	2603	10823	
10	4032	14208	
11	4402	9230	
12	3321	4536	
16	4640	6380	800 c.c.
17	1887	8769	
18	4108	11060	
19	2816	9472	
23	3108	4956	1000 c.c.
24	2304	16704	
25	2907	13680	
26	3944	8976	
27	3948	4032	
29	3404	5244	
30	4191	7747	
31	2838	5332	
Feb. 1	3780	6190	
2	5320	7700	
5	3408	3337	
6	2945	12190	
7	5075	14007	
8	4270	7198	
9	3741	4089	
10	3910	4165	
	Intravenous injection.		
March 12	4300	5200	1000 c.c.
13	1665	16835	
14	2940	16464	

When we consider the absolute values of the non-granular and polynuclear cells detailed above, it will be evident that the leucocyte response is mainly a polynuclear one. The total mononuclears fall suddenly, but rapidly regain their normal level, while the polynuclears rise slowly to a maximum and then slowly decline. From the fluctuations in the eosinophile and mast-cells it is impossible to draw any reliable inferences.

Summary and Conclusions.

It must be clear then from the above data on the horse and goat that the inoculation of diphtheria toxin produces a definite leucocytic reaction of polynuclear type. In fact the detailed evidence speaks more clearly in favour of a leucocytic reaction than Besredka's own results on the goat which he immunised. The question then arises: How are we to interpret the contradictory conclusions reached by Nicolas and Courmont? Metchnikoff (1905), while referring to the work of these authors in his *Immunity in Infectious Diseases,* maintains that a slight leucocytic reaction is apparent from their own figures and especially from the figures obtained in the early hours succeeding an injection. Of course, Nicolas and Courmont considered these slight changes to be negligible.

It seems to me, that the reason must be sought in the method of immunisation employed.

Nicolas and Courmont immunised their animals with very small doses many times repeated, and by such a method it was doubtless possible to reduce local swelling to a minimum. With no local reaction it would seem quite reasonable to expect an absence of general leucocytic reaction, but, as Metchnikoff maintains, such local tumefaction is never absent in horses which are subjected to increasing doses of toxin and which ultimately develop high grade antitoxin.

Throughout the immunisation of the animals detailed in the foregoing paper local swelling and oedema invariably followed the injections except in the earlier stages of immunisation when small doses were being given.

There is no doubt that, in order to produce a high degree of immunity in an animal, the stimulation must be sufficient. A certain small degree of immunity may always be reached without much leucocytic change, as was apparent in the early stages of "Plug's" immunisation.

It must be admitted, however, that, though the leucocytic reaction may be extremely marked after large doses of toxin, it does not necessarily follow that the antitoxin development will be correspondingly influenced.

Such a phenomenon was apparent in the case of "Plug," a relatively refractory horse.

In the more responsive horses, a parallelism between leucocytic reaction after large doses and increased antitoxin development would certainly be expected, without, however, inferring a causal relationship between these two phenomena. We cannot therefore admit the general applicability of Nicolas and Courmont's views, which merely indicate that cells subjected to small oft-repeated doses lose their power of reaction.

On the other hand there is probably no justification for the criticism brought forward by Besredka with regard to the results of Nicolas and Courmont, viz. that in the absence of antitoxin development, an absence of leucocytic reaction was to be expected.

We may therefore conclude that the leucocytic reaction of polynuclear type which follows the injection of large and increasing doses of diphtheria toxin is merely an evidence of efficient cell-stimulation and may not necessarily be accompanied by increased antitoxin development.

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ON THE LIQUEFACTION OF GELATIN BY THE BACILLUS CLOACAE.

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In the examination of a drinking water the chemist has a great advantage over the bacteriologist in that he can begin and complete his analysis in one day. The bacteriologist, on the other hand, is retarded in his work by several causes, one of which is that certain organisms liquefy gelatin only very slowly. The *Bacillus cloacae*, for instance, may not show any change in this medium for 30—40 days or even more. It is obvious that a delay of this duration may render a report valueless for practical purposes. Besides, the differentiation of *B. cloacae* from *B. lactis aerogenes* hinges upon the former's power of movement and of liquefying gelatin. It seemed, therefore, important to endeavour to devise some means by which it could be determined in a few days whether an organism belonged to the class of liquefiers. Various devices were tried, such as frequent sub-cultivation on various media prior to inoculation on to gelatin, the use of low percentages of gelatin, incubating at 37° C., or 42° C., and combinations of these, but all without success. It was, however, observed that old gelatin cultures which showed a stratum of liquefied medium about one quarter of an inch deep, became completely liquefied during a sojourn of 48 hours at 37° C., though they solidified on cooling if they had been kept only 24 hours at this temperature.

While I was talking over this point with Dr George Dean he suggested that it might be a question of the quantity of the organism or of the products of its growth used for inoculation which determined the time of commencement and completion of liquefaction, and for this suggestion I desire to thank him heartily as it has enabled me to make a step forward towards the solution of this problem.

In a previous number of this *Journal* (1905, Vol. v. p. 372), reference was made to certain bacilli of the *B. cloacae* group which took from six weeks to three months to liquefy $\frac{1}{4}$—$\frac{1}{2}$-inch of a gelatin tube. These partially liquefied tubes were placed at 37° C. for 48 hours, and the now completely liquid cultures were used to make fresh gelatin stab cultures (a loopful being used for each stab) which were kept at room temperature. At the end of 30 days there was no apparent liquefaction but in 40 days the liquefaction had extended about $\frac{1}{4}$-inch down the tube. A stay of 48 hours at 37° C. rendered the liquefaction complete.

Experiment I.

In this experiment three of these liquid tubes were used to inoculate two series of fresh gelatin tubes; Series I, consisting of three tubes, each of which received 1·0 c.c. of the liquid culture; and Series II, also of three tubes, each of which received 0·5 c.c. of the corresponding culture. Both series were incubated at 37° C., together with control tubes of uninoculated medium. Each day they were removed from the thermostat and placed in cold running water for 2—4 hours, during which period they were examined from time to time. If the gelatin set the tubes were at once returned to the incubator, but if at the end of four hours it was still liquid the tubes were left on the laboratory bench until the close of the experiment. This procedure brought out an interesting point in connection with the liquefaction of gelatin. It was noticed that some tubes which remained liquid after having been in cold water at 14—15° C. for four hours, became solid by the next morning when allowed to remain in a rack on the laboratory bench all night; the temperature of the room at the time of observation being about 19° C. This behaviour suggests the existence of a phase of delayed setting as a preliminary to complete liquefaction. In the results of all the following experiments this point has been taken into consideration and no tube has been considered to be liquefied unless it remained permanently liquid.

Results of Experiment I.

	Day:—1	2	3	4	5	6	7
Series I:							
1 c.c. in each tube	S	L	—	—	—	—	—
Series II:							
0·5 c.c. in each tube	S	S	S	S	semi-S	more liquid	L

S = solid on cooling. L = liquid on cooling.

Thus we see that 1 c.c. of an old liquefied gelatin culture of a *B. cloacae* liquefied 8—9 c.c. of gelatin in 48 hours at 37° C., while 0·5 c.c. took seven days to produce the same effect; and consequently that we cannot entirely leave out of consideration the part played by quantity in the production of this reaction.

Experiment II. To ascertain whether agar cultures would act like old gelatin cultures.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and another similar bottle with *B. lactis aerogenes*. Both bottles were placed at 37° C. After six days' incubation the growths were swept off the agar and each emulsified in 10 c.c. of sterile NaCl (0·85 %) solution. A gelatin tube was inoculated with 0·5 c.c. of the emulsion of *B. cloacae* and another tube with the like amount of the emulsion of *B. lactis aerogenes*. Both tubes were incubated at 37° C., and each day they were treated with cold water as in the previous experiment.

Result :

	Days :—5th	6th	9th	10th	15th
B. cloacae, 0·5 c.c.	not quite firm at R.T. 21° C.	semi-solid at 20° C. R.T.	semi-solid at 16° C. C.W.T.	liquid at C.W.T. 16·5° C.	—
B. lactis aerogenes, 0·5 c.c.	solid at R.T.	solid at R.T.	solid at R.T.	solid at R.T.	no sign of liquefaction

R.T. = room temperature. C.W.T. = cold water temperature.

The surface of the medium in a Roux bottle[1] equals that of 15—20 tubes containing sloped agar, therefore 0·5 c.c. of the emulsion, the amount of culture used for inoculation, would equal about one six-day agar tube. This amount liquefied 8—9 c.c. of ordinary nutrient gelatin in 10 days and was, therefore, not so powerful as the 0·5 c.c. of the old gelatin culture used in Exp. I.

Experiment III. To ascertain the effect of differences in the quantity and thickness of the emulsion.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. for six days, when the growth was swept off, emulsified in sterile NaCl (0·85 %) solution (the whole emulsion measured about 10 c.c.) and centrifugalised. Gelatin tubes were inoculated with 1 c.c., 0·5 c.c., and 0·25 c.c. respectively of the slightly turbid supernatant fluid, and then the remainder was thoroughly stirred up to form again a thick emulsion, of which 1 c.c., 0·5 c.c., and 0·25 c.c. were put into gelatin tubes. Both sets of tubes were incubated at 37° C., and cooled each day as in previous experiments.

[1] The agar in these Roux bottles is solidified while the bottle lies flat on its side. The agar surface thus obtained equals in area that of 15—20 ordinary slant agar tubes.

Result:

Material used for inoculation	Amount	Day:—3	5	7	11
Thick Emulsion	1 c.c.	solid on cooling	liquid at 14·5° C.	-	—
,,	0·5 c.c.	,,	,,	—	—
,,	0·25 c.c.	,,	solid	liquid at 14·5° C.	—
Turbid Supernatant fluid	1·0 c.c.	,,	solid	,,	—
,,	0·5 c.c.	,,	solid	,,	—
,,	0·25 c.c.	,,	solid	solid	liquid at 14·5° C.

This shows a difference in the time of liquefaction due to differences in the amount of material inoculated. The fact that the supernatant fluid liquefied the gelatin so soon suggests that the NaCl solution dissolved something which assisted in the solution of the gelatin.

Experiment IV. To ascertain the effect of young agar cultures in large quantity and also of a temperature of 37° C. *compared with the temperature of the room.*

A series of slant agar tubes were inoculated all over the surface with the *B. cloacae* and a similar series with *B. lactis aerogenes.* After 24 hours' incubation at 37° C., the growths were scraped off and gelatin tubes inoculated and incubated as recorded in the following Table (p. 27), which also gives the results.

On the 42nd day tube H was put into the 37° C. incubator. After 24 hours: solid on cooling; after 48 hours: liquid on cooling.

From these observations we must conclude that large inoculations by themselves are not of very great use but that they must be assisted by a temperature of 37° C. to obtain the best results.

Experiment V. To ascertain the effect of still larger inoculations of young agar cultures.

Slant agar tubes were inoculated with *B. cloacae*, the inoculating material being spread over the entire surface of the agar. After 24 hours' growth at 37° C., the growth was scraped off and used for inoculating tubes containing 8—9 c.c. nutrient gelatin which were then incubated at 37° C., and cooled down each day as before.

Result:

		Day:—2	3	4	5	6
Tube I	inoculated with the growth from 6 agar tubes	solid on cooling	solid on cooling	liquid	—	—
Tube II	inoculated with the growth from 3 agar tubes	,,	,,	not quite solid	semi-solid	liquid

Thus by using very large inoculations we can still further shorten the time required for liquefaction.

Table relating to Experiment IV.

	Amount of growth used for inoculation	Incubation temp.	Day :– 6	8	9	11	26	37	42
A.	1 loopful *B. lactis aerogenes*	37° C.	solid at 20° C.	solid at 19·5° C.	solid at R.T.	solid at R.T.	solid	solid	solid
B.	3 tubes *B. lactis aerogenes*	,,	,,	solid at R.T. 19·5° C.	,,	,,	,,	,,	,,
C.	1 loopful *B. cloacae*	,,	,,	solid at R.T. 19·5° C.	,,		liquid at 19° C. R.T.		
D.	3 tubes *B. cloacae*	,,	not quite firm	liq. at 19·5° semi-solid at 16·5° C.	liq. at C.W.T. 16·5° C.	—	—	—	—
E.	1 loopful *B. lactis aerogenes*	R.T.	solid	solid at R.T. 19·5° C.	solid at R.T.	solid	solid	solid	solid
F.	3 tubes *B. lactis aerogenes*	,,	,,	,,	,,	,,	,,	,,	,,
G.	1 loopful *B. cloacae*	,,	,,	,,	,,	,,	slight softening at one spot	3—4 drops of liquid gelatin	—
H.	3 tubes *B. cloacae*	,,	,,	,,	,,	small spot of softened gelatin	—	—	about ¼-inch of liquid gelatin

R.T. = room temperature. C.W.T. = cold water temperature.

Experiment VI. To ascertain the effect of lowering the percentage of gelatin.

A Roux bottle of nutrient agar (=15—20 agar tubes) was inoculated with *B. cloacae*, incubated at 37° C. for 24 hours, and then at room temperature for 4 days. The growth was then swept off and emulsified in salt solution (0·85 %). The emulsion measured from 14—15 c.c.

Two series of 12 tubes each were prepared, each tube containing 8 to 9 c.c. of nutrient gelatin. In Series I the percentage of gelatin in the medium was 5 % and in Series II 10 %. Into each tube was put 0·5 c.c. of the emulsion of *B. cloacae*. The tubes were sealed off in the flame to prevent evaporation, and incubated at 37° C. Beginning with Series I a tube was taken out of the incubator each day and cooled in water down to 13° C., the rest of the tubes being left at 37° C.

Of the tubes of Series I (5 % gelatin) the first to become liquid was that taken out of the incubator on the 8th day. As this one remained permanently liquid the remaining tubes of this series were cooled down and all were found to be liquefied. As all the tubes of Series I were completely liquefied a tube of Series II was also examined on this day and found to solidify on cooling.

The first tube of Series II (10 % gelatin) to show any sign of liquefaction was the tube removed from the incubator on the 12th day. This was not quite solid on cooling. The 14th day tube was liquefied and so were the rest of this series. The results are shown in the following Table.

	% gelatin	Day:—1	2	3	4	5	6	7	8	9	10	11	12	13	14
Series I	5	S	S	S	S	S	S	S	L	—	—	—	—	—	—
Series II	10	,,	,,	,,	,,	,,	,,	,,	S	S	S	S	S	S	L

S = solid. L = liquid.

Thus we see that the time of liquefaction can be shortened by using lower percentages of gelatin than the usual 10 %, a five-day agar growth liquefying 8—9 c.c. of 5 % gelatin in eight days, while it required 14 days to do the same in the case of 10 % gelatin.

Experiment VII. To ascertain the effect of large inoculations upon nutrient 5 % gelatin.

Inoculations were made with *B. cloacae* all over the surface of slant agar tubes which were incubated at 37° C. for 48 hours. The growth was then scraped off and used for inoculating tubes of nutrient 5 % gelatin. The incubation temperature was 37° C., and the tubes were examined each day as before.

No. of tube	Amount inoculated	Day:—1	2	3	4
1.	Growth from 6 agar tubes	Both solid at R.T. 13° C.	Both not quite solid after 2 hours at R.T. 13° C.	Both liquid at R.T. 13° C.	Both liquid at the temp. of cold water 10° C.
2.	,, ,, 3 ,, ,,			very viscid after 3 hrs. at C.W.T. 9° C.	

R.T. = room temperature. C.W.T. = cold water temperature.

If we compare these results with those obtained with 10 % gelatin we find there is a slight advantage in using the less solid medium. It must be borne in mind that in all these experiments it is the time of complete liquefaction which has been noted. If one had been content only to cool the inoculated tubes down to a temperature at which control uninoculated tubes became solid the power of liquefaction could have been made evident at an earlier period than is shown in the tables.

The experiments detailed above have shown that by means of massive inoculations into 5 % gelatin and by incubating at 37° C., the power possessed by *B. cloacae* of liquefying gelatin can be demonstrated in a much shorter time than by the usual method.

The solution of the gelatin is no doubt due to a ferment such as has been proved by Bitter, Fermi, Hueppe, and others[1] to be a product of the metabolism of many organisms. Only two experiments in connection with this point will be given here, all questions as to its mode of action, etc., being left for discussion on a future occasion.

Experiment VIII. To ascertain whether cultures of B. cloacae killed by chloroform liquefy gelatin.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. for 48 hours. The growth was then washed off with 10 c.c. of sterile salt solution and emulsified. A few drops of chloroform were now added, the mouth of the bottle was plugged tightly with wool and covered with a lead capsule and the bottle then placed at 37° C. for 48 hours. The whole emulsion measured 18 c.c. Tubes containing about 8 c.c. of gelatin were inoculated thus :

No. 1	with	5 c.c.	of emulsion			
,, 2	,,	4	,,	+1 c.c.	of sterile tap	water
,, 3	,,	3	,,	+2	,,	,,
,, 4	,,	2	,,	+3	,,	,,
,, 5	,,	1	,,	+4	,,	,,
,, 6	,,	1	,,			
,, 7	,,	1·5	,, .			

The sterile tap water was added to make the volume of fluid added equal in the case of the first five tubes. The tubes were placed in the incubator at 37° C., and were examined and cooled in water each day as in previous experiments.

After 24 *hours* at 37° C., all the tubes soon became solid when allowed to remain at the temperature of the room.

On the second day tubes 6 and 7 became solid after about 15 minutes at room temperature but in the remainder the setting was very much delayed and was not absolutely complete in 4 hours.

[1] Cited by Kolle and Wassermann, *Handbuch der pathogenen Micro-organismen*, Bd. I. 1903, p. 106.

On the third day 6 and 7 solid at room temperature; the others liquid. After 1 hour in cold water at 10° C.: No. 2 almost set. After 2 hours at 10° C.: No. 4 set, Nos. 1 and 5 almost quite solid, No. 3 liquid, except for about ¾-inch at the bottom of the tube where the gelatin was too solid to pour out. On this day a loopful of the liquid gelatin was taken out of tubes 1—5 and used to inoculate slant agar tubes which, after 48 hours' incubation at 37° C., showed no growth. Thus it may be accepted that tubes 1—5 did not contain any living organisms.

On the fifth day No. 6 solid at room temperature, 11·5° C., Nos. 1—5 and 7 liquid after 1½ hours in a rack at room temperature. After 3 hours at 9° C. cold water temperature: 7 a viscid liquid, and the others liquid. Left at room temperature overnight; they all remained liquid. These results are tabulated below.

Results of Experiment:

No. of tube	Material inoculated	1st day	2nd day	3rd day	5th day
1.	5 c.c. of emulsion of *B. cloacae*	solid R.T.	solid R.T.	liquid R.T. solid 10° C.	liquid at 9° C.
2.	4 ,, ,, +1 c.c. sterile water	,,	,,	,,	,,
3.	3 ,, ,, +2 ,, ,,	,,	,,	,,	,,
4.	2 ,, ,, +3 ,, ,,	,,	,,	,,	,,
5.	1 ,, ,, +4 ,, ,,	,,	,,	,,	,,
6.	1 ,, ,,	,,	,,	solid R.T.	solid R.T.[1] 11·5° C.
7.	1·5 ,, ,,	,,	,,	,,	liquid at 9° C.

R.T. = room temperature.

It is thus evident that the liquefaction of gelatin is due to a ferment. It was expected that by using varying amounts of material for inoculation corresponding differences in the time required for liquefaction would be shown, but this has not been the case, and no explanation of the fact is at present forthcoming. It must be borne in mind that in measuring out an emulsion of this kind it is impossible to make certain of obtaining an equal amount of effective material in each cubic centimetre. But this will not account for the difference between tubes 5 and 6, which each received as nearly as possible the same amount of emulsion and contained the same quantity of material to be peptonised. Here the only explanation appears to lie in the different degrees of concentration of the medium in the two tubes. That this is not the entire explanation however is shown by the following experiment:

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. At the end of 48 hours the growth was scraped off and emulsified in 10 c.c. of distilled water. The whole emulsion measured 15 c.c. It was poured into a sterile flask, a few drops of chloroform added, the flask tightly corked and placed

[1] On the 8th day this tube remained liquid at R.T. = 15° C.

Table relating to Experiment VIII.

Tube	Amount of material inoculated	Day :– 1	2	3	4	5	7	8
1.	3 c.c. of emulsion + 3 c.c. sterile salt solution	Solid on cooling 10° C.	S 10° C. C.W.T.	L 10° C.	—	—	—	—
2.	2 ,, ,, + 4 ,, ,, ,,	,,	,,	,,	—	—	—	—
3.	1 ,, ,, + 5 ,, ,, ,,	,,	,,	,,	—	—	—	—
4.	0·5 ,, ,, + 5·5 ,, ,, ,,	,,	,,	,,	—	—	—	—
5.	0·25 ,, ,, + 5·75 ,, ,, ,,	,,	,,	S 10° C.	S 10° C.	L 10° C.	—	—
6.	3 ,, ,, only	,,	L 10° C.	—	—	—	—	—
7.	2 ,, ,, ,,	,,	,,	—	—	—	—	—
8.	1 ,, ,, ,,	,,	S 10° C.	L 10° C.	—	—	—	—
9.	0·5 ,, ,, ,,	,,	,,	S 10° C.	L 10° C.	—	—	—
10.	0·25 ,, ,, ,,	,,	,,	,,	S 10° C.	S 10° C.	S 9° C. C.W.T.	L 10° C.

C.W.T. = cold water temperature.

S = solid.
L = liquid.

at 37° C. After allowing autolysis to proceed for 48 hours the emulsion was used to inoculate tubes containing 6—6·5 c.c. of 10 % nutrient gelatin as in the preceding Table (p. 31).

With the idea of obviating the sedimentation and consequent unequal distribution of the organisms throughout the emulsion which takes place when measuring out emulsions with the usual large bore 10 c.c. pipette all the varying quantities of emulsion in this experiment were measured with the same 1 c.c. pipette. The tubes were incubated, etc., as before.

As soon as a tube became liquid, inoculations were made from it on to agar to ascertain whether the tubes were free from living organisms. Growth was obtained in the case of tubes 3 and 5, but as the organisms did not give the reactions of *B. cloacae* and did not liquefy gelatin their presence had no special influence on the result. The $\frac{\text{time}}{\text{quantity}}$ relation comes out fairly well in the series of tubes 6—10, but it cannot be said that dilution of the gelatin has had a very marked effect in tubes 1—5. We would not, therefore, be justified in saying that the rapidity of liquefaction of gelatin by this enzyme is proportional to the degree of concentration of the medium.

Summary.

The *Bacillus cloacae* liquefies gelatin very slowly, sometimes taking a month or more to do so. It is in consequence occasionally a source of delay and of great inconvenience to the bacteriologist. In this paper it has been shown that by using appropriate methods the time necessary for demonstrating the liquefaction of gelatin by this organism may be shortened to a week.

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A CONTRIBUTION TO THE BACTERIOLOGY OF MILK.

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In a previous number of this *Journal* (1905, vol. v. p. 333) reference was made to certain organisms isolated from samples of town milk sold for domestic use. The examination was undertaken for the purpose of ascertaining whether the lactose fermenting organisms present were of the same varieties, and in the same group-proportions as those in cow faeces. This should be, presumably, the case if the statement is correct that it is practically impossible under ordinary conditions to obtain milk uncontaminated with cow-dung. The constant presence of such pollution has been suggested by Park (1901, p. 400) and others. Delépine (1903) considers that "it is even difficult to conceive how slight faecal pollution of cow's milk can entirely be prevented under any circumstance"; and Boekhout and de Vries (1904) are of the same opinion, for they say: "Es wohl kaum Milch geben wird welche nicht mit kleinen Mengen von Kuhfaeces verunreinigt ist." The results given in my previous paper did not lead to a definite conclusion on this point. It was thought, therefore, that it would be of interest to repeat the experiments with country milk as sold for domestic use, and with milk obtained direct from the udder, as thus any change due to bacterial growth during the interval between collection and examination would be minimised or entirely avoided. The technique employed was practically the same as in the previous experiments except that after isolation the organisms were subjected to a greater number of tests. Cultures in gelatin were incubated at room temperature; those in other media were kept at 37° C.

Description of samples and of methods used to isolate the organisms.

Sample 1 was obtained at a farm. It was a poor milk and not clean. Two to three c.c. were put into a tube of bile salt inulin broth and incubated at 37° C. overnight. Next day surface cultivations were made on bile salt neutral-red lactose agar plates. After 18—24 hours' growth at 37° C. cultures were made from 13 different colonies, and the organisms thus isolated tested as described below.

Sample 2 was also obtained at a farm and was the mixed milk of several cows. It was excellent milk and very clean. The procedure was exactly the same as in the case of Sample 1, but cultures were taken from only 10 colonies.

Samples 3, 4, 5 *and* 6 were obtained in the street from carts belonging to four different dealers who sell milk in this neighbourhood. They were ordinary samples and were examined in the same manner as the two previous samples. The number of colonies sub-cultured was respectively 13, 10, 10 and 10.

Samples 7 *and* 8 were really only one sample, that is to say, they were portions of the same mixed milk but the method used for isolation was different. In the case of Sample 7, 20 c.c. of milk were centrifugalized for 6 hours and the deposit was spread over the surface of bile salt lactose agar plates. In the case of Sample 8 the procedure was the same as for Samples 1 to 6. The idea of treating the two portions differently was to ascertain whether preliminary incubation would markedly alter the result. The number of colonies fished were 10 and 11 respectively.

Table I shows that the organisms are practically the same in both cases.

Sample 9 was a mixed milk. Twenty c.c. were centrifugalized for 6 hours and plates made both from the creamy upper layer and from the sediment, 3 plates from each. Next day the plates made from the sediment showed only small round, non-acid colonies consisting of elliptical cells resembling a small yeast. The plates from the creamy layer showed many similar small non-acid colonies and 11 acid colonies. All the latter were sub-cultured.

Samples 10, 11 *and* 12 were portions of a mixed milk. No. 10, Twenty c.c. were centrifugalized for 6 hours and plates made from the sediment and creamy layer mixed. No. 11, 2 c.c. were put into a tube of bile salt mannite broth and incubated at 37° C. for 6 hours, when plates were made as before. The broth tube (now called No. 12) was then returned to the incubator, left at blood temperature over-night and next morning a loopful of the culture was spread on the surface of plates. Ten colonies were sub-cultured from each set of plates, and each of these 30 colonies was found to be formed by the same variety of bacillus.

The next six samples were from a single cow. The milk was drawn from all the teats into sterile tubes, at the beginning, in the middle, and at the end of milking, to see if the varieties of lactose fermenters varied with the stage in the process of milking at which the milk was collected. They run in pairs, Nos. 13 and 16 being portions of the first milk, Nos. 14 and 17 of the middle milk, and Nos. 15 and 18 of the end milk. The corresponding members of each pair were treated in the same way.

Samples 13, 14 *and* 15. Three c.c. put into a sterile test-tube and placed at 37° C. for 18 hours, when plates were made as before on bile salt lactose agar. Thirty-five

colonies were sub-cultured from these three sets of plates. The organisms were all staphylococci (orange, white and yellow), and with one exception, all failed to liquefy gelatin in 6 weeks.

These samples were left on the laboratory bench for 48 hours. There was then no smell whatever, and no apparent change in the milk. A drop from each tube put on litmus paper showed a faint trace of acidity.

Samples 16, 17 *and* 18. Three c.c. were put into a tube of bile salt dulcit broth and kept in ice-chest overnight and then incubated at 37° C. for 6 hours when plates were made as previously. At the end of 48 hours' incubation there was no growth. The dulcit broth tubes were then put in the incubator for 24 hours when plates were again made from them. Only cocci were present. Four days later plates were once more made from these broth tubes. No growth was obtained.

Samples 19, 20 *and* 21 were portions of a single milk drawn into sterile tubes at the commencement, middle, and end of milking respectively. Each sample was treated in the same way. A dilution of 1 in 100 of sterile water was made and tubes of nutrient broth were inoculated with 0·1 c.c., 0·25 c.c., 0·5 c.c., 0·75 c.c. and 1 c.c. of this dilution. Tubes of broth were also inoculated with 0·05 c.c. and 0·1 c.c. of undiluted milk. Tubes of bile salt mannite broth were inoculated with 0·05 c.c., 0·1 c.c. and 1 c.c. of undiluted milk. 10 c.c. of undiluted milk were also put into each of 5 sterile test-tubes. All tubes were placed at 37° C.

Next day inoculations were made on slant agar from all the tubes except those containing 10 c.c. of undiluted milk. From one of these latter a loopful was spread over plates of ordinary lactose agar and of bile salt lactose agar, 3 plates being made from each loop and thus 6 from each sample. All tubes and plates were incubated at 37° C. Inoculations into mannite broth were made later from each 10 c.c. tube.

The results of the examination were as follows:

Sample 19. Growth in 1/100th of a c.c. but not in 1/130th c.c. Only non-liquefying staphylococci were found in all tubes up to 1 c.c. when bacilli were observed to be present. *No gas-forming organisms were found in* 50 c.c.

Sample 20. Growth in 1/20th c.c. tube but not either in 1/10th c.c. and in 1/100th c.c. Only non-liquefying staphylococci in 1 c.c. Bacilli were first found in 10 c.c. *No gas-forming organisms were present in* 50 c.c.

Sample 21. Growth in 1/20th c.c. but not in 1/100th. Non-liquefying staphylococci and a bacillus which produced a yellow pigment were isolated. *No gas-forming organisms were present in* 50 c.c.

Sample 22. Portions of the whole milking from a single cow: the milk was drawn straight from the udder into a sterile test-tube, and examined in the same way as the three previous samples except that the inoculations into bile salt mannite broth were omitted. The incuba-

tion temperature was 37° C. Non-liquefying staphylococci were found in 1/100th c.c. but not in 1/130th c.c. Bacilli were first noticed in 10 c.c. *No gas-forming organisms were found in the* 50 c.c. *examined.*

Sample 23. A portion of the same milk as No. 22 taken from the ordinary milking pail. It was treated as No. 22. Non-liquefying staphylococci were present in 1/130th c.c. but not in 1/200th c.c. The further results were exactly as in the case of No. 22.

Sample 24 was the same milk as No. 23, but after it had been passed through the straining cloth. It was examined in the same way as Nos. 22 and 23. Growth was obtained from the smallest quantity examined, *i.e.*, 1/1000th c.c. . Bacilli, and these were liquefiers, occurred first in 1/200th c.c. Gas-forming organisms did not appear until the 10 c.c. tubes were examined. Plates were made from one of these tubes and 10 colonies were sub-cultured.

A sample of the water in which the milk-pails were usually washed was also analysed. The amounts examined were 1/10,000th, 1/1000th, 1/100th, 1/10th, 1, 10 and 250 c.c. The incubation temperature was, as before, 37° C. A growth was obtained from 10 c.c. but not from 1 c.c. Plates were made and 10 pure cultures obtained.

The time of milking of Samples 1, 3, 4, 5 and 6 was not ascertained but was presumably the early morning of the day of examination and, therefore, the analysis was commenced within 5 hours from the time of collection. All the other samples were obtained at one farm. Nos. 2, 7, 8, 9, 10, 11 and 12 were milk as sold at the farm from the 6 a.m. milking and the examination was commenced at about 10 a.m. In the case of Nos. 13 to 24 the cows were milked from 3.30 to 4 p.m., the samples were brought straight to the laboratory and the analysis commenced at once. The cows were milked by the farm people in the manner usual at this farm and beyond collecting the milk samples in sterile tubes no precautions were taken to avoid contamination.

Though gas-forming organisms were not present in Samples 13—23, and though the number of samples is so small, still it may be of interest to compare the results with those of some other workers.

Thus Park (1901, p. 393) found the average bacterial content of the milk from 6 separate cows, examined 5 hours after collection, to be 6000 per c.c., the lowest count being 400 per c.c., and that of 25 cows tested immediately after milking to be 4550. He used agar plates as "nutrient gelatin was found to be more troublesome and not to yield more accurate results than nutrient agar." Two sets of plates were

made. One set was incubated at about 20° C. and counted on the 4th day; the other was put at 37° C. for 24 hours, and then for a second 24 hours at 20° C. He states that from 5—30 % more colonies developed on the plates incubated at room temperature than on those kept at 37° C. for 24 hours. Thus, to compare his lowest count with my results we must deduct 30 % from the 400 which gives 280 per c.c. as his lowest count in the case of milk from a single cow and "when every reasonable means was taken to insure cleanliness."

Burr (1902), also taking every reasonable precaution, found 500 organisms per c.c. in the milk of a single cow. Making, for the purposes of comparison, the same deduction as before we get the number 350 per c.c.

Ed. v. Freudenreich (1902) thought it would be easy to carry out strict asepsis and thus obtain a bacteria-free milk. But he soon came to the conclusion that this was impossible. He found that the milk always contained 200—300 organisms per c.c. even though the milker's hands and the teats were washed first with soft soap and sterile water, then with servatol soap and sterile water, finally with sterile water alone and dried on a sterile towel; the milker's hands were smeared with lanoline, and the first milk rejected. After all these precautions had been taken the milk was drawn into a sterile funnel and thus into a sterile flask. Twenty-eight cows were milked in this way. The bacterial content of the mixed milk of these 28 cows varied from 65—680 organisms per c.c. in 18 samples:

65	per c.c.	in	1	sample,
100—200	„	„	8	samples,
200—300	„	„	3	„
300—400	„	„	5	„
680	„	„	1	sample.

The mean was 230 per c.c. Some single flasks were examined and yielded from 55—125 organisms per c.c.

He found non-liquefying cocci in greatest numbers. Bacteria were few.

Freudenreich and Thoni (1903) from a further series of similar experiments conclude that freshly drawn milk, even when the most careful precautions are taken against contamination, always contains bacteria, that these are mostly cocci, and that they come from the udder.

Continuing his experiments v. Freudenreich (1903) states that he

examined the udders and the milk in the udders of 15 cows; in 13 cases immediately after slaughtering. He found none bacteria-free. The organisms were mostly cocci. *B. lactis acidi* was met with once only. In three cases the ducts were diseased and in these cases the diseased tissues contained fewer organisms than usual. *B. coli* was never found.

He mentions that Boekhout and de Vries drew milk directly from the udder with a sterile cannula and always got a growth from it.

Lux (1904) examined milk which was drawn in the manner customary in the place. No antiseptic precautions were used. The milk was drawn into sterile test-tubes which were not allowed to touch the teat, and the plug of which was only removed for the few seconds during which the milk was flowing. 260 cow-milk and 95 goat-milk samples were analysed. The medium used was whey agar, the incubation temperature 38° C., and the count was made on the fourth day. The average per c.c. was 1395 organisms which were mostly non-pathogenic cocci.

Henderson (1904) examined 7 normal udders and obtained growth in 76 % of the cultures made, the organisms being staphylococci, streptococci and pseudo-diphtheria bacilli. None of the organisms were found to be pathogenic to laboratory animals. Two of these normal udders were from young heifers, and were unexpanded, and all the cultures made from the milk cistern, ducts and parenchyma remained sterile.

Willem and Miele (1905) obtained a milk containing 2·5 bacteria per c.c. The milking was done in a special place which was kept as aseptic as possible. The greatest care was taken to ensure the cows being clean. The udder and teats were washed before each milking with soap and boiled water or an antiseptic solution.

From the examples quoted we see that it is impossible to obtain bacteria-free milk but that the organisms in carefully collected milk are not pathogenic to the usual laboratory animals. We may allow then that the presence of such organisms in reasonable number would not render a milk harmful to man. Lux's experiments have shown that with very ordinary care it is possible to obtain a milk containing on an average 1400 bacteria per c.c. and it is obvious that with very slight trouble the number may be much reduced.

The work of Park (1901), Nicolle and Petit (1903), Conn and Esten (1904), Koning (1905), F. C. Harrison (1905), and others, has shown that if milk be rapidly cooled to 11° C. or below, very little, if any multiplication of organisms takes place for some 12 hours. Therefore

Park's suggested average standard of not more than 12,000 bacteria per c.c. in warm, and 5000 per c.c. in cold weather, seems a generous standard and one which with a little care should be easily attained.

It is necessary to note that "Separator-milk" must not be judged by the same standard as fresh milk, for Severin and Budinoff (1905), and Severin (1905), have shown that even when every possible precaution is taken against contamination the milk issuing from the separator always contains many more bacteria than it did before it passed into the separating-chamber. Severin suggests that the mechanical movement completes the separation of bacteria which were only partially divided when they entered the machine.

It may be of interest to mention that Jensen (1903) states that the results of the Copenhagen Hygienic Commission showed that of 142 samples of pasteurised milk 98 samples contained more than 100,000 microbes per c.c.

Tests applied to the organisms isolated.

All the gas-forming bacilli isolated from the samples described were studied with regard to the following characters:

(1) Morphology. 24-hour agar cultures were used.

(2) Motility. Two separate cultures of each of these organisms were examined microscopically for the presence or absence of motility. On the first occasion 24-hour broth cultures were examined by the hanging drop method with a $\frac{1}{6}$th objective and a $\times 8$ eyepiece. On the second occasion young broth cultures, about six hours old, were used. A drop of the culture was placed on a slide and examined under dark ground illumination with a $\frac{1}{2}$ in. objective and a $\times 8$ eyepiece. The results were the same in each case. I found the second method the easier and to give in my opinion a better idea of the power of locomotion. Any organism was considered to be motile when there was distinct translation from place to place. No observations were made as to whether there were rotatory or other movements without progressive motion.

(3) Staining by Gram's method.

(4) Production of indol—broth cultures, about 8 to 10 days old, were used. About 1 c.c. of a 0·02 % solution of sodium nitrite was added to each tube and then a few drops of sulphuric acid.

(5) Voges and Proskauer's reaction.

(6) Gas percentage.

(7) Ratio of H to CO_2.

Fermentation tubes containing 2 % peptone (Witte) and 1 % glucose in tap-water were inoculated with the various organisms and incubated at 37° C. After 48 hours growth they were taken out of the incubator, allowed to cool, and the amount of gas produced noted. The bulb was then filled with 2 % NaHO solution, the opening closed with the thumb and the tube thoroughly shaken. After all the gas had been collected in the closed arm the thumb was removed and air allowed to enter the bulb to replace any CO_2 absorbed. The ratio of H to CO_2 was then observed and the tubes allowed to stand at room temperature for 48 hours when the presence or absence of Voges and Proskauer's reaction was noted[1].

(8) Growth of nutrient agar.

(9) „ „ „ gelatin.

(10) „ „ „ broth.

(11) „ „ litmus milk.

(12) Fermentation of various carbo-hydrates, alcohols, etc.

Characters of the organisms isolated.

The organisms isolated all had the following characters in common. They were all Gram-negative, non-sporing, bacilli of much the same size and shape and without any special morphological differences. The growths on agar and gelatin were all non-characteristic. Both gelatin stab, and slope cultures were made. Some of the stab cultures showed a

[1] I have given all the details of the technique employed because in a recent number of the *Bulletin de l'Institut Pasteur*, vol. IV. 1906, p. 250, there is a note to the effect that MacL. Harris has found Voges and Proskauer's reaction to be given by every organism and that, therefore, it is of no value as a distinguishing characteristic. No details of Harris's technique are given. Since reading that note I have re-tested some 20 of the organisms described in this paper. The cultures were incubated for 5 days at 37° C. and after adding the NaHO allowed to stand for a week: the results were similar to those previously obtained, *i.e.* certain organisms gave the reaction and others did not. It is, however, quite possible that this reaction may be quantitative and not qualitative and that the substance, acetylmethylcarbinol (Harden, 1906) necessary for the appearance of the red colour may be produced by all bacteria which decompose glucose, but that the quantity produced by some of them from a 1 % solution of glucose in 48 hours may be too small to give rise to any visible colouration on the addition of NaHO.

I can only say that under the conditions given above I have found this reaction of great use in the differentiation of these lactose fermenting bacilli.

wrinkled surface growth in a week to 10 days but wrinkling was not observed in any of the slope cultures. All produced general turbidity in nutrient broth. In every case litmus milk was acidified and coagulated. All produced acid and gas in glucose, lactose, mannite, sorbit, but not a single one had any effect on erythrit.

The remaining reactions are given in the following Table:

Table I.

No. of Sample	No. of organism	Cane Sugar	Dulcit	Adonit	Inulin	Gas percentage in Glucose	H/CO_2	V & P reaction	Indol	Liqn. of gelatin	Motility
1	1	–	–	–	–	30	4/1	–	+	–	–
	2	–	–	+	–	20	4/1	–	+	–	–
	3	–	–	–	–	45	3/1	–	+	–	–
	4	–	–	–	–	25	4/1	–	+	–	–
	5	+	–	+	–	25	5/1	–	+	–	–
	6	+	+	+	–	40	3/1	–	+	–	+
	7	–	+	–	–	30	4/1	–	+	–	–
	8	+	–	+	–	45	3/1	–	+	–	–
	9	+	+	+	–	20	5/1	–	+	–	+
	10	+	–	+	–	25	5/1	–	+	–	–
	11	–	+	–	–	45	5/1	–	+	–	–
	12	+	–	+	–	30	5/1	–	+	–	–
	13	–	–	–	–	45	3/1	–	+	–	–
2	14	–	+	–	–	25	5/1	–	+	–	–
	15	–	+	–	–	30	5/1	–	+	–	+
	16	–	–	–	–	45	2/1	–	+	–	–
	17	–	+	–	–	20	5/1	–	+	–	–
	18	–	+	–	–	45	3/1	–	+	–	–
	19	–	+	–	–	30	4/1	–	+	–	–
	20	–	+	–	–	11	3/1	–	+	–	–
	21	–	+	–	–	25	3/1	–	+	–	+
	22	–	–	–	–	25	4/1	–	+	–	–
	23	–	+	–	–	40	2/1	–	+	–	–
3	24	+	–	–	–	90	1/2	+	–	+	+
	25	+	–	+	–	50	2/1	–	–	–	–
	26	+	–	+	–	55	2/1	–	–	–	–
	27	+	–	–	–	90	1/2	+	–	+	+
	28	+	–	–	–	90	1/2	+	–	+	+
	29	–	–	+	–	40	3/1	–	+	–	+
	30	+	+	+	+	90	1/1	+	+	–	–
	31	–	+	–	–	60	2/1	–	–	–	+
	32	–	+	–	–	50	2/1	–	–	–	+
	33	–	+	–	–	50	2/1	–	–	–	+
	34	+	–	–	–	90	1/2	+	–	+	+
	35_1	–	–	–	–	60	2/1	–	+	–	–
	35_2	+	–	+	+	30	3/2	+	+	–	–

TABLE I. (*continued*).

No. of Sample	No. of organism	Cane Sugar	Dulcit	Adonit	Inulin	Gas percentage in Glucose	H/CO_2	V & P reaction	Indol	Liqn. of gelatin	Motility
4	36	+	–	–	–	90	2/3	+	–	+	+
	37	+	–	–	–	90	1/2	+	–	+	+
	38	+	–	–	–	80	2/3	+	–	+	+
	39	+	–	–	–	90	1/1	+	–	+	+
	40	+	–	–	–	80	1/1	+	–	+	+
	41	+	–	+	+	30	2/1	+	–	–	–
	42	+	–	+	–	60	3/2	–	–	–	–
	43	+	–	–	–	60	1/2	+	–	+	+
	44	+	+	+	+	80	1/1	+	–	–	–
	45	+	+	+	+	70	2/1	+	–	–	–
5	46	+	–	+	–	100	1/2	+	–	–	–
	47	+	+	+	–	75	3/2	+	–	–	–
	48	+	–	+	–	90	2/3	+	–	–	–
	49	–	+	–	–	50	3/1	–	+	–	+
	50	–	+	–	–	65	2/1	–	+	–	+
	51	–	+	–	–	45	3/1	–	+	–	+
	52	–	+	–	–	50	3/1	–	+	–	+
	53	–	+	–	–	50	3/1	–	+	–	+
	54	–	+	–	–	50	3/1	–	+	–	+
	55	–	+	–	–	25	3/1	–	+	–	+
6	56	–	+	–	–	40	2/1	–	+	–	–
	57	–	+	–	–	40	7/1	–	+	–	–
	58	+	+	–	–	30	3/1	–	+	–	–
	59	–	+	–	–	50	3/1	–	+	–	–
	60	–	+	–	–	50	3/1	–	+	–	–
	61	–	+	–	–	45	3/1	–	+	–	–
	62	–	+	–	–	20	3/1	–	+	–	–
	63	–	+	–	–	40	3/1	–	+	–	–
	64	–	+	–	–	50	3/1	–	+	–	–
	65	–	+	–	–	30	2/1	–	+	–	–
7	66	–	+	–	–	50	2/1	–	+	–	–
	67	+	+	+	+	90	1/1	+	+	–	–
	68	+	+	+	+	90	1/1	+	+	–	–
	69	–	+	–	–	30	2/1	–	+	–	–
	70	–	+	–	–	40	2/1	–	+	–	–
	71	–	+	–	–	25	3/1	–	+	–	–
	72	–	+	–	–	25	3/1	–	+	–	–
	73	–	+	–	–	25	5/1	–	+	–	–
	74	+	+	–	–	40	2/1	–	+	–	–
	75	+	+	–	–	50	2/1	–	+	–	–
8	76	–	+	–	–	40	4/1	–	+	–	–
	77	–	+	–	–	25	2/1	–	+	–	–
	78	–	+	–	–	20	2/1	–	+	–	–
	79	–	+	–	–	40	2/1	–	+	–	–
	80	+	+	–	–	40	2/1	–	+	–	–
	81	–	+	–	–	50	2/1	–	+	–	–
	82	+	+	–	–	50	2/1	–	+	–	–

Table I. (*continued*).

No. of Sample	No. of organism	Cane Sugar	Dulcit	Adonit	Inulin	Gas percentage in Glucose	H/CO_2	V & P reaction	Indol	Liqn. of gelatin	Motility
8	83	–	+	–	–	25	3/1	–	+	–	–
	84	–	+	–	–	25	2/1	–	+	–	–
	85	+	+	+	+	80	3/2	+	+	–	–
	86[1]	–	–	–	–	20	2/1	–	+	–	+
						[1] Lactose –					
9	87	+	+	+	+	70	1/1	+	+	–	–
	88	+	+	+	+	90	1/1	+	+	–	–
	89	+	+	+	+	25	2/1	+	+	–	–
	90	+	+	+	+	80	1/1	+	+	–	–
	91	+	+	+	+	85	1/1	+	+	–	–
	92	+	+	+	+	80	1/1	+	+	–	–
	93	+	+	+	+	95	1/1	+	+	–	–
	94	+	+	+	+	80	1/1	+	+	–	–
	95	+	+	–	–	50	2/1	–	+	–	+
	96	+	+	+	+	70	1/1	+	+	–	–
	97	+	+	+	+	80	1/1	+	+	–	–
10	98–107	–	+	–	–	10–25	2–4/1	–	+	+	+ only in the cold
11	108–117										
12	118–127										
	Lactose and mannite were fermented very slowly—7 days. Cultures white at first. Yellow pigment developed in 7 days.										
13	128–140	All non-liquefying staphylococci yellow, white, and orange, except No. 159 which was an orange liquefying staphylococcus.									
14	141–150										
15	151–160										
24	161	+	+	–	–	50	3/2	–	+	–	–
	162	+	+	–	–	50	3/2	–	+	–	–
	163	+	+	–	–	55	3/2	–	+	–	–
	164	+	+	–	–	50	3/2	–	+	–	–
	165	+	+	–	–	60	5/4	–	+	–	–
	166	+	+	–	–	45	3/2	–	+	–	–
	167	+	+	–	–	60	5/4	–	+	–	–
	168	+	+	–	–	60	5/4	–	+	–	–
	169	+	+	–	–	55	5/4	–	+	–	–
	170	+	+	–	–	50	3/1	–	+	–	–
Water	1	–	–	–	–	65	5/4	–	–	–	–
	2	–	–	–	–	40	5/4	–	–	–	–
	3	–	–	–	–	55	5/4	–	–	–	–
	4	–	–	–	–	55	5/4	–	–	–	–
	5	–	–	–	–	55	5/4	–	–	–	–
	6	–	–	–	–	50	5/4	–	–	–	–
	7	–	–	–	–	55	3/2	–	–	–	–
	8	–	–	–	–	60	3/2	–	–	–	–
	9	–	–	–	–	50	3/2	–	–	–	–
	10	–	–	–	–	55	3/2	–	–	–	–

+ = the production of acid and gas, or of Voges and Proskauer's reaction, or of indol, or of liquefaction of gelatin, or the presence of motility as the case may be.

– = a negative result.

V & P = Voges and Proskauer's "Kalilaugeroth-reaction."

These may, as in my previous article, be arranged in groups according to the action they have on cane sugar and dulcit.

Group I = Both cane sugar and dulcit unaffected.
" II = Cane sugar unaffected—dulcit fermented.
" III = Both cane sugar and dulcit fermented.
" IV = Cane sugar fermented—dulcit unaffected.

No. of Sample	Group I	Group II	Group III	Group IV	No. of organisms isolated
1	5	2	2	4	13
2	2	8	—	—	10
3	2	3	1	7	13
4	—	—	2	8	10
5	—	7	1	2	10
6	—	9	1	—	10
7	—	6	4	—	10
8	—	7	3	—	10
9	—	—	11	—	11
24	—	—	10	—	10
	9	42	35	21	107

If these numbers be calculated as percentages and compared with the numbers previously obtained with cow faeces (MacConkey, 1905) a parallelism in the group percentages is at once apparent,

Country Milks	8·4 %	39·2 %	32·7 %	19·6 %
Cow faeces	16·6	25·0	47·9	12·5

and it is obvious that Groups II and III form the majority in both cases.

Again, if we arrange these samples according to the opportunities which have presumably occurred for contamination to take place and then calculate the group percentages, an interesting point is brought out:

Samples	Group I	Group II	Group III	Group IV	Total No. of organisms isolated
2, 7, 8, 9, 24	3·9 %	41·1 %	54·9 %	—	51
1, 3, 4, 5, 6	12·5	37·5	12·5	37·5 %	56
Town milk	32·5	15	20	32·5	40

From which it would seem that the farther we get from the source the greater the change in the group proportions. Thus nearest the source we find Groups II and III taken together forming 96 % of the organisms isolated. A short distance away they form 50 %, and still further away the number decreases to 35 %. While Groups I and IV increase from 3·9 % at the source to 65 % at the point farthest away.

Having thus considered these organisms as members of various groups we may now study them individually and compare them with certain known organisms whose reactions have been worked out and gathered together in Table II. This list is longer than the one

Table II.

	Cane Sugar	Dulcit	Adonit	Inulin	Gas percentage in Glucose	H/CO_2	V & P	Indol	Liqn. of gelatin	Motility
B. Grünthal	–	–	–	–	50	3/1	–	+	–	+
B. levans	–	–	–	+	60	1/2	+	–	+	+
B. acidi lactici (Hüppe), Král	–	–	+	–	30	2/1	–	+	–	–
B. acidi lactici (Hüppe), St George's	–	–	+	–	15	3/1	–	+	–	–
B. coli communis	–	+	–	–	25	2/1	–	+	–	+
B. cavicida, (Brieger) Pasteur	–	+	–	–	20	3/1	–	+	–	+
B. cavicida, (Brieger) Král	–	+	–	–	25	3/1	–	+	–	+
B. mustelae septicus	–	+	–	–	25	3/1	–	+	–	+
B. Neapolitanus, Král	+	+	–	–	30	2/1	–	+	–	–
B. rhinoscleromatis, W.	+	+	+	–	40	3/1	–	+	–	–
,, ,, R.	+	+	+	–	40	3/1	–	+	–	–
B. pneumoniae (Friedländer), N.	+	+	+	–	30	3/1	–	+	–	–
B. pneumoniae (Friedländer), Nic.	+	+	+	–	30	3/1	–	+	–	–
B. pneumoniae (Friedländer), A.	+	+	+	–	35	3/1	–	+	–	–
B. pneumoniae (Friedländer), Král	+	+	+	–	35	3/1	–	+	–	–
B. pneumoniae (Friedländer), Allen	+	+	+	–	35	3/1	–	+	–	–
B. oxytocus perniciosus, Král	+	+	+	+	60	1/1	+	–	–	–
B. lactis aerogenes	+	–	+	–	25[1]	2/1[1]	+	+	–	–
B. capsulatus (Pfeiffer), Pasteur	+	–	+	–	25[1]	2/1[1]	+	+	–	–
B. capsulatus (Pfeiffer), Král	+	–	+	–	60[1]	2/3[1]	+	+	–	–
B. cloacae (Jordan), Jordan	+	–	–	–	75	1/1	+	–	+	+
B. cloacae (Jordan), Král	+	–	–	–	50	1/1	+	–	+	+
B. coscoroba	+	–	–	–	40	3/1	–	+	–	–

+ = production of acid and gas, of Voges and Proskauer's reaction, or of indol, or of liquefaction of gelatin, or the presence of motility as the case may be.

– = a negative result.

V & P = Voges and Proskauer's "Kalilaugeroth-reaction."

[1] On this occasion *B. lactis aerogenes* and *B. capsulatus* (Pfeiffer), Pasteur, gave results which were smaller than those given by *B. capsulatus* (Pfeiffer), Král, which gave the usual percentage and ratio.

previously given and I beg to thank Dr J. Binot of the Pasteur Institute, Paris, for so kindly sending me the following cultures: *B. rhinoscleromatis* W., do. R., *B. pneumoniae* (Friedländer), W., do. Nic., do. A., *B. cavicida* (Brieger), *B. capsulatus* (Pfeiffer), *B. coscoroba.* To Prof. E. O. Jordan I am greatly indebted for a culture of *B. cloacae*, to Dr H. de R. Morgan for *B. Grünthal*, to Dr R. M. Allen for *B. Friedländer* (Allen) and to Dr H. Spitta for *B. acidi lactici* (Hüppe), St George's. From Král's laboratory I obtained *B. cavicida* (Brieger), K., *B. levans, B. mustelae septicus, B. oxytocus perniciosus.*

As *B. levans, B. coscoroba* and *B. oxytocus perniciosus* are not so well known as most of the others the following notes may be of interest.

Holliger (1902) cites Wolffin (1894) as describing the *B. levans* to be the cause of the fermentation of dough and to be in all probability identical with *B. coli.* According to Wolffin's description the *B. levans* is :—"A facultative anaerobic rod $1{\cdot}8\mu \times 0{\cdot}6\mu$. No threads are formed in cultures. It is non-sporing. In most cultures it is indistinguishable from *B. coli* but it does not produce indol, and does not coagulate milk. It ferments dextrose with a ratio of $H : CO_2 = 1 : 3$. Lactose is not attacked." However, both F. Frankel and J. Papasotiriu found that it produced indol and caused acidity and coagulation in milk.

Holliger found two bacilli in fermenting dough : (1) A motile liquefying bacillus which produced a yellow pigment ; and (2) A non-chromogenic liquefying bacillus. Both fermented dextrose and lactose and coagulate milk. The rate of liquefaction varied very much ; the white bacillus sometimes taking 1—2 months. No. 1 resembled No. 2 in that at first the growths were not yellow. He compared these bacilli with a *B. levans* from Král's laboratory and found it and the white bacillus to be identical. The ratio of H to CO_2 was as 1 to 2. Indol was formed by all three.

The yellow bacillus found in samples 10, 11 and 12 agrees in many respects with Holliger's yellow bacillus.

B. coscoroba (*Ann. de l'Inst. Pasteur*, 1900) is stated to be the cause of an epidemic which occurred in a particular race of swans, "Cygne de Coscoroba."

B. oxytocus perniciosus Wyssokowitsch. A reference to this bacillus may be found in Teisi Matzuschita's *Bakteriologische Diagnostik*, 1902, p. 452. He states that it was isolated from stale milk.

All these organisms fermented glucose, lactose, galactose, laevulose, mannose, arabinose, raffinose, mannite, sorbit, dextrin.

Erythrit was not attacked in one single case.

They all produced acidity and caused clotting in litmus milk. In the case of the Friedländer bacilli and the two *B. rhinoscleromatis* the coagulation occurred very late, *i.e.* in 14 to 21 days.

It has been suggested that organisms may vary so much as regards their fermentative powers that these reactions are of only slight, if any, value

as differentiating characteristics. Therefore before proceeding further it is necessary to consider briefly the question of how much reliance may be placed upon these fermentation tests as a means of differentiating and identifying these organisms. It is true that it is not an uncommon laboratory experience to find that an organism if left uncultivated for some time has lost part of its fermenting power. Such loss, however, is not permanent and a few sub-cultures soon restore the former vigour. The cause of such temporary weakening has not been discovered and seems inexplicable in view of the unfavourable conditions to which organisms have been subjected without loss, even temporary, of power. Thus, as I have previously shown, *B. coli communis* can be kept under most unfavourable conditions for about 1 year without losing any of its vigour. The following experiment also bears upon this point. On November 1st, 1905, I inoculated a gelatin tube with *B. cloacae* and sealed up the tube in the flame. The culture was placed at 37° C. for about 24 hours and then left on the laboratory bench in a glass jar exposed to diffuse daylight from a north window until May 11th, 1906, *i.e.*, over six months. An examination of the tube on this date showed that the gelatin was completely liquefied and was perfectly clear and transparent above the growth deposited at the bottom of the tube. After shaking to diffuse the growth throughout the gelatin sub-cultures were made in various media and it was found that the fermentation reactions were just the same as before and took place with just as much vigour, showing that an autolysis of six months duration had not impaired the vitality of the bacillus.

Again, if these fermentative reactions are so very variable we should expect to find that different strains of the same organism, which had been kept in different laboratories and most likely grown on media of varying composition for varying lengths of time, would show differences in their behaviour to various fermentable substances. But this does not appear to be the case. A glance at Table II will show that all the Friedländer bacilli react alike; both *B. cloacae* act in the same way; identical results are given by the two strains of *B. acidi lactici* (Hüppe), and so on. Further, I tested 15 different races of the *B. typhosus* with regard to this point. The ages of the strains varied very much. One had been grown on artificial media for 16 years, another for 10 years, and the others for varying lengths of time; the youngest having been isolated from a typhoid stool only a week or two previously. All these 15 races behaved exactly alike as regards the reactions tested. Now, if a strain 16 years old does not differ from one only a week or two

old, surely these characters may be considered to be comparatively stable.

We may also consider the question from another point of view. We may start with the postulate that a substance which is decomposed by a large number is a more easily fermentable substance than one which is split up by only a small number of organisms. Now, if we examine Table I we find that dulcit was fermented by 77 organisms, cane sugar by 56, and adonit by 32. Therefore, we would be probably justified in presuming that bacteria find adonit more difficult to decompose than either dulcit or cane sugar. Consequently, if an organism fermented all these substances and, owing to unfavourable environment or any other cause, began to lose some of its vigour, we should expect the power of fermenting adonit to fail first. The reaction of a certain number of the organisms in the Tables will be found to uphold this hypothesis but, on the other hand, many will be found to contradict it, and the *B. acidi lactici* is an example of a bacillus which, on this hypothesis, has retained the power of fermenting the most stable substance while it has lost the power of attacking the less stable bodies.

If we entertain the opposite hypothesis and consider that all the positive reactions are acquired, we are brought up by the *B. levans* which would then have acquired the power of fermenting inulin before that of acting upon dulcit, cane sugar, or adonit.

Besides, on one of these hypotheses we should expect to find organisms like the *B. Grünthal* most numerous whereas such is not the case in these experiments; nor did it occur in my previous ones, where out of 480 lactose fermenters only 25 per cent. belonged to the first group. Or, to take a more special case, out of 75 organisms isolated from time to time out of a mixture of human faeces and tap-water only 6 did not attack either cane sugar or dulcit, and these 6 were found in the early part of the experiment, whereas, if the "loss of power" hypothesis be correct, the number of these organisms should have shown an increase as the experiment progressed and the environment became more and more unfavourable, and at the end should have reached a larger proportion than 6 out of 75.

Again, we may suppose the fermentative powers of these bacilli to be very variable and we may apply a number of tests to them. In these circumstances we would expect to obtain as many varieties of bacilli as there are combinations of tests. The following Tables show the number of possible combinations of the tests applied and consequently the number of possible varieties of bacilli, together with the varieties actually obtained.

Group I. Cane sugar = – Dulcit = –	Adonit +	Inulin +	V & P +	Motility +		
				Motility –		
			V & P –	Motility +		
				Motility –		
		Inulin –	V & P +	Motility +		
				Motility –		
			V & P –	Motility +	29	
				Motility –	2	*B. acidi lactici* (Hüppe).
	Adonit –	Inulin +	V & P +	Motility +		
				Motility –		
			V & P –	Motility +		
				Motility –		
		Inulin –	V & P +	Motility +		
				Motility –		
			V & P –	Motility +	*B. Grünthal.*	
				Motility –	1, 3, 4, 13, 16, 22, 35_1.	
Group II. Cane sugar = – Dulcit = +	Adonit +	Inulin +	V & P +	Motility +		
				Motility –		
			V & P –	Motility +		
				Motility –		
		Inulin –	V & P +	Motility +		
				Motility –		
			V & P –	Motility +		
				Motility –		
	Adonit –	Inulin +	V & P +	Motility +		
				Motility –		
			V & P –	Motility +		
				Motility –		
		Inulin –	V & P +	Motility +		
				Motility –		
			V & P –	Motility +	15, 21, 31, 32, 33, 49, 50, 51, 52, 53, 54, 55, *B. coli communis*, *B. cavicida*, *B. mustelae septicus*.	
				Motility –	7, 11, 14, 17, 18, 19, 20, 23, 56, 57, 59, 60, 61, 62, 63, 64, 65, 66, 69, 70, 71, 72, 73, 76, 77, 78, 79, 81, 83, 84.	

Note:—The numbers refer to the organisms in Table I.

Group III. Cane sugar = + Dulcit = +	Adonit +	Inulin +	V & P +	Motility +	
				Motility −	30, 44, 45, 67, 68, 85, 87, 88, 89, 90, 91, 92, 93, 94, 96, 97, *B. oxytocus perniciosus.*
			V & P −	Motility +	
				Motility −	
		Inulin −	V & P +	Motility +	
				Motility −	47
			V & P −	Motility +	6, 9.
				Motility −	*B. pneumoniae* (Friedländer), *B. rhinoscleromatis.*
	Adonit −	Inulin +	V & P +	Motility +	
				Motility −	
			V & P −	Motility +	
				Motility −	
		Inulin −	V & P +	Motility +	
				Motility −	
			V & P −	Motility +	95.
				Motility −	58, 74, 75, 80, 82, 161–170, *B. neapolitanus.*
Group IV. Cane sugar = + Dulcit = −	Adonit +	Inulin +	V & P +	Motility +	
				Motility −	35_2, 41.
			V & P −	Motility +	
				Motility −	
		Inulin −	V & P +	Motility +	
				Motility −	46, 48, *B. lactis aerogenes*, *B. capsulatus* (Pfeiffer).
			V & P −	Motility +	
				Motility −	5, 8, 10, 12, 25, 26, 42.
	Adonit −	Inulin +	V & P +	Motility +	
				Motility −	
			V & P −	Motility +	
				Motility −	
		Inulin −	V & P +	Motility +	1 1 1 1 1 1 1 1 1 1
				Motility −	24, 27, 28, 34, 36, 37, 38, 39, 40, 43, *B. cloacae.*
			V & P −	Motility +	
				Motility −	*B. coscoroba.*

1 = liquefier of gelatin.

We find that the number of varieties isolated falls very far short of the number possible in the circumstances. The most striking result is shown in Group II where 42 organisms are divided into only two varieties and these differ from each other with regard to one point only, *i.e.* the presence or absence of motility.

All these points which I have mentioned appear to me to be greatly in favour of accepting fermentation reactions as a means of differentiating and identifying these organisms.

Proceeding on these lines it will be found that of the known organisms the

B. acidi lactici (Hüppe)	has been	isolated	once,
B. coli communis	„	„	12 times,
B. neapolitanus	„	„	15 „
B. lactis aerogenes	„	„	twice,
B. cloacae	„	„	10 times,
B. oxytocus perniciosus	„	„	16 „

while the *B. Grünthal, B. pneumoniae* (Friedländer), and *B. coscoroba* have not been met with at all.

It must be carefully noted that all the samples which contained gas-forming organisms were milks which had been drawn into one vessel and then passed through a strainer into another vessel and possibly into several others before being sold. There was, therefore, plenty of opportunity for contamination to occur. It is precisely in those samples which, as far as one can judge, were most exposed to contamination that we find *B. lactis aerogenes* and *B. cloacae.*

In the samples drawn direct from the cow no gas-forming organisms were met with. These results agree on most points with those obtained by other workers. v. Freudenreich (1902) expresses the opinion that in milk drawn direct into sterile tubes one usually finds only cocci present. v. Freudenreich and Thoni (1903) conclude that lactic acid bacilli are not found in freshly drawn milk. v. Freudenreich (1903) never found *B. coli* in the examination of 15 udders and of the milk in them. v. Freudenreich (1904) never found *B. coli* or *B. lactis aerogenes* in the udder and only once in milk taken direct into sterile glasses. In the latter case he thinks it was a contamination. Moreover he did not test the gas-producing power of this organism. He mentions the interesting fact that he found on his plates *coli*-like and *aerogenes*-like colonies which were formed of cocci, and also *aerogenes*-like colonies of a non-gas-forming bacterium, and *coli*-like colonies of a bacterium which did not ferment milk sugar.

Lux (1904) found *B. coli* and *B. lactis aerogenes* taken together in 9% of his samples. But he took no precautions as regards cleaning the udder.

Burr (1902) instituted several series of experiments to ascertain the source of the acid organisms of milk and cream. He took great care to have the stables, cows, and everything connected with them thoroughly clean. In milk drawn straight into test-tubes he did not find lactic acid bacilli but they were found in the cream of milk drawn into a sterile pail (cf. Sample 9). Plates exposed under the cows for 10, 20, 30 and 40 seconds during milking all contained *B. lactis aerogenes, B. acidi lactici* II, but no *B. acidi lactici* I. Tubes of sterile milk exposed about the barn and under the cows contained in nearly every case all the varieties of bacilli mentioned, while milk taken at the same time direct from the udder did not contain any. He also examined the glandular part, the milk cistern, and the beginning of the teat of two udders, and failed to find any of these three bacilli.

He concludes that the lactic acid organisms, *B. lactis aerogenes, B. acidi lactici* II and 1—the latter organism being the *B. acidi lactici* of Hüppe, Marpmann and Esten,—are a contamination from outside the udder. *B. acidi lactici* II and *B. lactis aerogenes* are commonly very abundant in the stable, while *B. acidi lactici* I, though almost universally present, exists in relatively small numbers.

Conn (1902) considers that the milk bacteria come seldom from the milk passages but chiefly from sources distinct from the cow.

Harrison (1905) examined the milk of 25 cows. The udders and flanks were brushed and wiped with a damp cloth, the first milk was rejected and the samples then taken in sterile test-tubes. From the milk of two of these cows gas-producing bacteria were isolated. He gives the characteristics of seven organisms isolated from the udder. In the case of six of them the ratio $\frac{H}{CO_2} = \frac{1}{1+}$ and cane sugar was fermented. Two of these were motile so that it is not at all unlikely they were *B. cloacae.* He mentions that Moor and Ward have also isolated gas-producing bacilli from the udders of certain cows but that it is evidently an exceptional state of affairs. Altogether he isolated 48 gas-producing organisms from various samples of milk. 21 of these gave a gas ratio of $\frac{1}{1}$ or $\frac{1}{1+}$ and 37 fermented cane sugar. Of these latter five were motile.

Savage (1906) as the result of his investigations found that milk

from individual cows yielded *B. coli* in 17·5 % of the samples. In 11 mixed milks collected fresh at the farm and examined within three hours *B. coli* was present in 36 %, and in 16 shop samples and mixed milk samples, not examined at once, they were present in 94 % of the samples.

Heinemann (1905) considers that the lactic ferments are of intestinal origin and are present in milk owing to contamination with faeces.

It seems then to be the general opinion that gas-forming bacteria are not normally present in milk but that they gain access to it because of want of care and cleanliness during the milking and in connection with the various vessels in which the milk is stored. This opinion is quite borne out by the results of the experiments detailed in this paper.

Summary.

With ordinary care and cleanliness it is possible to obtain milk which, when freshly drawn, contains less than 1500 organisms per cubic centimetre.

Freshly drawn milk should not contain gas-forming organisms in at least 50 c.c.

Gas-forming organisms are present in milk owing to contamination with faecal material. Of these organisms the *B. oxytocus perniciosus*, the *B. neapolitanus* and the *B. coli communis* occur in greatest number in fresh milk, while the *B. cloacae* and *B. lactis aerogenes* appear at a later stage.

Out of 107 non-chromogenic lactose fermenters isolated from milk only one bacillus gave the reactions of the *B. acidi lactici* (Hüppe); while the *B. Grünthal*, *B. pneumoniae* (Friedländer), and the *B. coscoroba* have not been met with once.

Finally, I desire to enter a plea for the substitution of certain fermentation reactions in the place of some of the tests now usually employed, in the routine examination of water and food-stuffs, to identify these organisms. At present bacteriologists make use of some, or all, of the following tests:

1. Morphology.
2. Motility.
3. Staining by Gram's method.
4. Character of the growth on nutrient gelatin.

5. Liquefaction of gelatin.
6. Action on milk.
7. Formation of indol.
8. Fermentation of glucose.
9. „ „ lactose.
10. „ „ cane sugar.
11. Action on neutral-red.

All the organisms mentioned in Tables I and II produced clotting in milk, fermented glucose, gave the neutral-red reaction, had much the same morphology, and the growths on agar and gelatin did not afford any assistance towards differentiating between them.

I would therefore suggest that instead of five of the tests, Nos. 4, 6, 7, 8 and 11, we should substitute the following:

Fermentation of dulcit,
„ „ adonit,
„ „ inulin,
Voges and Proskauer's reaction,

as by so doing we should get a finer differentiation without increase in work and we should not be classing as *B. coli* organisms which may have little in common with, and have a distribution entirely different from that of the *B. coli communis.*

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NOTE ON SOME CASES OF FOOD-POISONING.

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The cases which form the subject of the following note occurred in the district of Dr Chas. Jackson, M.O.H., of Fulham, and I am greatly indebted to him for so kindly furnishing me with their histories and with samples of the patients' serum.

There were in all four cases, the patients being a man (*A*), his wife (*B*), baby (aged four months) (*C*), and grandmother (*D*). On Saturday, September 15th, 1902, *A* bought three rabbits, one of which he gave to his grandmother, who cooked it and ate it the next day without any ill effects. The other two rabbits were stewed on the Sunday and partaken of by *A* and *B* for dinner and supper without any untoward results. What was left over from Sunday was warmed up on the Monday for dinner and supper, *D* being present at the latter meal. On this occasion *C* had a small quantity of the gravy. The wife (*B*) was seized with diarrhoea and vomiting the same evening, and the others early the following morning. In the cases of *A*, *B*, and *D*, the symptoms were severe but passed off under treatment. The baby's illness, beginning with diarrhoea and vomiting, passed into collapse and finally into coma, which after three days' duration ended in death on the seventh day of the attack. A post-mortem examination was made four days after death. The materials sent to me for examination consisted of a piece of the infant's spleen (Sept. 26) and a portion of the hind-limb of a rabbit (Sept. 21). The surface of the latter was black and charred, and I understood that it was picked out of the ashpan underneath the fire, where it had been thrown after supper.

The method of examination was the same in each instance. A portion of the tissue from the inside of the specimen was removed, put into a tube of bile salt glucose broth (every precaution being taken to avoid accidental contamination), and placed at 37° C. After 24 hours'

incubation, surface cultivations were made from the glucose broth on to bile salt neutral-red lactose agar plates which were incubated at 37° C. From these plates pure cultures were obtained and a further examination showed that in both cases the same organism had been isolated. It was an actively motile bacillus, non-sporing, non-liquefying, and Gram negative. It produced non-characteristic grey-white growths on agar and gelatin. Milk became slightly acid at first but this soon gave place to distinct alkalinity. It fermented

Glucose,	Mannite,	Dextrin,
Mannose,	Dulcit[1],	
Maltose,	Sorbit,	
Arabinose,		
Raffinose[2],		

with the formation of acid and gas.

It had no action on

Lactose,	Adonit,	Inulin.
Cane sugar,	Erythrit,	

The cultural reactions thus corresponded in every particular with those of the *B. enteritidis* (Gaertner) group. Both bacilli were found to be pathogenic for guinea-pigs when injected subcutaneously. A monkey was fed (by a tube) with a 24 hour broth culture of the "rabbit

[1] *Dulcit.* This reaction is very valuable indeed. Its great importance as a differentiating characteristic was first pointed out by me at a laboratory meeting of the Pathological Society of London held on February 3rd, 1903.

[2] *Raffinose.* These bacilli, just like all the members of the *B. enteritidis* Gaertner group, fermented raffinose with the production of acid always, and often also of a small amount of gas. But there seems some doubt as to the nature of this reaction.

L. Telesmin (*Centralbl. f. Bakteriol.* Abt. II., Bd. XII. 205—216, 1904) states that Kahlbaum's raffinose, after having been heated to 100° C., reduces Fehling's solution, and that on hydrolysis it yields glucose, galactose and fructose. In view of this statement a medium was made up composed of peptone 2 % and gelatin 10 % in tap-water, the whole being rendered slightly alkaline to litmus. Kahlbaum's raffinose was dissolved in cold distilled water and the solution sterilised by filtration through a porcelain filter. Enough of this raffinose solution was added to the gelatin to make the amount of raffinose equal to 1 %. Neutral-red was then added as an indicator. The tubes were incubated to ascertain whether any contamination had occurred. As they were found to be sterile inoculations were made with about 15 different members of the group and all produced acid as evidenced by the change of colour of the neutral-red, a control tube remaining unchanged.

Messrs Merck, however, have informed me that there is no method of ascertaining whether a sample of raffinose is quite free from traces of fructose, etc. It would seem, therefore, that one cannot place much reliance upon this reaction.

bacillus" mixed with a similar culture of the "child's bacillus." It developed diarrhoea and died during the third night. Cultures were made in bile salt glucose broth from the heart blood, spleen, a mesenteric gland and faeces. No growth was obtained from the heart blood or spleen, but from the faeces and gland was isolated a bacillus corresponding in all cultural reactions with the bacilli used for feeding. Specimens of the patients' blood were obtained and tested as below:

1st examination.

	Dilution	Time	*B. typhosus*	*B. enteritidis* of Gaertner	Bacillus from rabbit
Blood of A	1/50	1 hour	+ +	+	0
,, B	1/50	2½ hours	+ +	0	0
,, D	1/50	2½ ,,	+ +	0	0

2nd examination.

Blood obtained 23 days after beginning of attack.

						Bacillus from child
Blood of A	1/50	2½ hours	+ +	0	0	+
,, B	1/50	2½ ,,	+ +	0	0	+
,, D	1/50	2½ ,,	+ +	+	+	+

A had had enteric fever four years previously.

+ + = good sized clumps but fair number of motile bacilli in field.

\+ = only small clumps: many motile bacilli in field.

Two rabbits were then immunised, one with the bacillus isolated from the piece of rabbit and the other with the bacillus obtained from the child's spleen. The serum of these rabbits was tested with the results shown in the following table. The dilutions of serum used were 1/20, 1/100, 1/500, 1/1000, 1/5000, 1/10000; the time limit was two hours, and the cultures were 24 hour growths in bouillon. The hanging

	Rabbit bacillus serum	Child bacillus serum
Bacillus from rabbit	1/1000	1/1000
,, ,, child	1/1000	1/1000
B. paratyphoid A (Schottmüller)	<1/20	1/20
,, B (,,)	1/1000	1/1000
B. L. (Hume)[1]	1/1000	1/1000
B. Aertrycke	1/1000	1/1000
B. "*Gl.*"[2]	1/500	1/1000
B. psittacosis (Nocard)	1/500	1/1000
B. enteritidis (Gaertner)	1/20	<1/20
B. icteroides (Sanarelli)	<1/20	1/20
B. Danysz	<1/20	1/20

[1] *Bacillus L.* (Hume) differs from the rest in that it ferments adonit with the production of acid and gas.

[2] *Bacillus* "*Gl.*" was isolated from the heart-blood of a guinea-pig which died after inoculation with the *Bacillus mallei*.

drop method was employed and observations were made with a 1/2 inch objective and × 8 eyepiece under dark background illumination. The results record the limit of dilution with which agglutination occurred as tested against a control. As soon as the mixture of serum and culture gave appearances similar to the control the result was noted as negative and the preceding dilution was entered as the limit of agglutination.

These agglutination reactions make it clear that the two bacilli isolated during these experiments are closely related to *B. Aertrycke* (van Ermengem) and *B. paratyphoid* "B" (Schottmüller) and are different from either *B. enteritidis* (Gaertner) or *B. paratyphoid* "A" (Schottmüller). They also show that the bacillus isolated from the heart-blood of a guinea-pig dead of glanders is, as far as it has been tested, identical with *B. psittacosis* (Nocard).

Conclusion.

The bacillus isolated from the piece of rabbit is identical with the bacillus obtained from the child's spleen and both belong to that group of organisms which has been most frequently associated with cases of food-poisoning. The attacks were caused by this bacillus as is shown by the effect of feeding a monkey with pure cultures. In about 60 hours these bacilli had passed out of the intestine and penetrated as far as the mesenteric glands but had not reached the general circulation.

The fact that all the cases occurred within an hour or two of each other and that the Monday's supper was the only meal at which the baby tasted the rabbit suggests that when purchased the rabbit was quite fit for human consumption, and contamination must have taken place in the consumer's house.

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Upon the Properties of an Antityphoid Serum obtained from the Goat.

By ALLAN MACFADYEN, M.D.

(Communicated by Dr. C. J. Martin, F.R.S. Received March 2,—Read March 8, 1906.)

(From the Bacteriological Department, Lister Institute.)

In this communication I propose to give a brief account of the results obtained by the immunisation of the goat with the cell juices of the *Bacillus typhosus*, in continuation of researches already published which had for their main object the production of an antibody for the endotoxin of the typhoid organism.* These experiments met with partial success and failure. It will be sufficient on the present occasion to say that the goat proved a more suitable animal than the horse for arriving at a solution of the theoretical and practical considerations involved. The object was to arrive at the best method of producing with the material employed an antiendotoxin in adequate amount, with a view to its reapplication to the horse.

During the progress of these researches experiments on analogous lines have been published by Dr. Besredka,† and to these I will, in the first instance, refer. Dr. Besredka describes the results following the intravenous injection into a horse of the intact dead and living typhoid bacilli. The injections of the bacilli were carried out for a period of two years. The serum was tested for antiendotoxic properties against (1) killed and dried cultures of the *B. typhosus*, of which 0·01 gramme killed guinea pigs on intraperitoneal injection; (2) a soluble endotoxin extracted from the dead and dried bacilli, of which 1/8 c.c. killed guinea pigs. The results were as follows: 10 or 15 centigrammes of the dried serum (about 1 c.c. and 1·5 c.c. before drying) neutralised 10 or 15 lethal doses of the killed typhoid culture respectively on intraperitoneal injection into the guinea pig, and the 10 centigrammes of the dried serum neutralised 16 lethal doses of the soluble

* "Upon the Immunising Effects of the Intracellular Contents of the Typhoid Bacillus, as obtained by the Disintegration of the Organism at the Temperature of Liquid Air," by Allan Macfadyen, 'Roy. Soc. Proc.,' March 12, 1903; "Upon the Intracellular Constituents of the Typhoid Bacillus," by Allan Macfadyen and Sydney Rowland, 'Centralblatt für Bakteriologie,' Abth. 1, vol. 30, 1901, No. 20; and vol. 34, 1903, Nos. 7 and 8.

† "Etudes sur le bacille typhique et le bacille de Peste," par Dr. Besredka, 'Annales de l'Institut Pasteur,' July 25, 1905: "De l'Antiendotoxine Typhique," par Dr. Besredka, *Ibid.*, February, 1906.

endotoxin. To obtain this low antitoxic value in the horse a period of two years was apparently necessary.

The typhoid cell juices employed in my experiments were prepared as follows: Virulent typhoid bacilli were cultivated on nutrient agar in Roux bottles for 18 hours at blood heat. The growth was brushed off and washed with distilled water in a centrifuge for half an hour. The bacilli were then triturated at the temperature of liquid air in the grinding pot already described by Rowland and myself.* The time allowed was 30 minutes per gramme of bacilli. The mass was taken up in 1/1000 solution of caustic potash, and centrifuged for two hours. The supernatant fluid was pipetted off, and represented a 10-per-cent. extract of the ground mass. This was treated with chloroform vapour for half an hour. The juices obtained under these conditions were sterile and toxic on intravenous injection into the test animals employed. The endocellular toxins obtained in this manner are unstable bodies, and the juices rapidly decrease in toxicity. The experiments with kept juices did not indicate that their injection would lead to any marked tolerance for fresh and markedly toxic juices: the probabilities were against this easier and less risky method of procedure. Resort was accordingly made to the use of fresh and acutely toxic juices, which contained on an average 10 to 12 milligrammes of solid matter per cubic centimetre. The fresh juices on intravenous injection were acutely toxic for the goat. The first goat died after the injection of 1 c.c., whilst 1/10 c.c. killed several animals. In two instances 1/20 c.c. killed within 12 hours. Death was preceded by profuse diarrhœa and collapse. Where death did not occur, the injection of 1/20 c.c. was followed by illness and diarrhœa, and 1/30 c.c. rendered certain animals ill, but with less acute symptoms. It was obvious that the intravenous injections would have to be carefully carried out to avoid unduly depressing or killing the animals.

A goat after receiving 1/20 and 1/10 c.c. cell juice died, whilst another, after the injection of doses of 1/20, 1/10, 1/2, and 1 c.c. at intervals of seven days, died within four hours after receiving the last injection. The indication, it appeared to me, was to start with small sublethal doses, and to raise them very gradually at duly spaced intervals. One injection weekly proved to be the safest procedure, and in the later experiments the same dose was repeated until it failed to produce toxic symptoms in the animal. A higher dose was then given and the process repeated. This method proved successful, as the animals became tolerant to otherwise fatal doses of the toxins.

In the experiments here recorded the antiendotoxic action of the serum

* 'Centralblatt f. Bakter.,' vol. 34, No. 7, 1903.

was tested by intravenous injection in rabbits. The endotoxin likewise killed rabbits acutely, *e.g.*, 1/10 c.c. killed with diarrhœa and collapse, and at times within two hours, whilst 1/20 c.c. was not infrequently a lethal dose. The onset of the toxic symptoms usually occurred in from one to two hours. The conditions were therefore sufficiently stringent in respect of any antitoxic action of the treated goats' serum. I satisfied myself that neither the goats' nor rabbits' serum possessed any appreciable antitoxic power for the toxins employed. Controls were subsequently found to be unnecessary. Three cubic centimetres of normal goat and rabbit serum did not neutralise two or ten lethal doses of the cell juices. The serum of the treated goats was tested against multiple lethal doses of the endotoxin. An estimate was likewise made of the agglutinative and bacteriolytic properties of the serum. I now proceed to an account of the first successful immunising experiment.

Billy Goat I.—Received following intravenous injections of toxic cell juices of *B. typhosus*:—

1905.					1905.				
May	16	1/20	c.c.	Ill.	July	7	1	c.c.	No symptoms.
„	24	1/10	„	Ill.	„	14	1·5	„	No symptoms.
June	2	1/5	„	Ill.	„	21	1·5	„	No symptoms.
„	9	1/2	„	Ill.	„	28	2	„	Ill.
„	16	1	„	Ill.	Aug.	4	2	„	No symptoms.
„	23	1	„	Ill.	„	11	2·5	„	Dead next day.
„	30	1	„	Ill.					

It was evident that with succeeding animals an even more careful system of dosage would have to be adopted. The goat was bled at intervals and its serum tested. In all instances the serum-toxin mixture was kept at 37° C. for 30 minutes previous to intravenous injection in the rabbit. The results are given in Table II.

I.—Test of Normal Goat's Serum.

Rabbit	I	3 c.c. serum	+1 c.c.	toxin.	Dead.
„	II	2 „	+1 „	„	Dead.
„	III	Control.	1/10 „	„	Dead.
„	IV	„	1/20 „	„	Diarrhœa.

Three cubic centimetres of normal serum did not neutralise 1 c.c. of a toxic cell juice of which 1/10 killed and 1/20 c.c. produced acute diarrhœa in the rabbit.

II.—Tests of Serum of Goat I.

Date.	Test animal.	Amounts injected.	Results.
June 23	Rabbit 1	3 c.c. serum + 1 c.c. toxin.	Alive.
,,	,, 2	Control, 1 c.c. toxin.	Dead.
July 7	,, 1	3 c.c. serum + 2 c.c. toxin.	Alive.
,,	,, 2	3 ,, + 1 ,,	,,
,,	,, 3	2 ,, + 1 ,,	,,
,,	,, 4	Control, 2 c.c. toxin.	Dead, 4 hours.
,,	,, 5	,, 1/20 ,,	,, 18 ,,
July 14	,, 1	3 c.c. serum + 2 c.c. toxin.	Alive.
,,	,, 2	2 ,, + 2 ,,	,,
,,	,, 3	1 ,, + 1 ,,	,,
,,	,, 4	1/10 ,, + 1 ,,	,,
,,	,, 5	Control, 1/10 c.c. toxin.	Dead.
,,	,, 6	,, 1/20 ,,	Diarrhœa and collapse.
July 21	,, 1	1 c.c. serum + 1 c.c. toxin.	Alive.
,,	,, 2	1/2 ,, + 1 ,,	,,
,,	,, 3	1/10 ,, + 1 ,,	,,
,,	,, 4	Control, 1 c.c. toxin.	Dead.
,,	,, 5	,, 1/10 ,,	Diarrhœa and collapse.
,,	,, 6	,, 1/15 ,,	,, ,, ,,
August 4	,, 1	1/20 c.c. serum + 1 c.c. toxin.	Alive.
,,	,, 2	1/50 ,, + 1 ,,	,,
,,	,, 3	1/100 ,, + 1 ,,	,,
		Control not made.	
August 11	,, 1	1/20 c.c. serum + 1 c.c. toxin.	,,
,,	,, 2	1/50 ,, + 1 ,,	,,
,,	,, 3	1/100 ,, + 1 ,,	Dead.
,,	,, 4	Control, 1 c.c. toxin.	,, 2 hours.
,,	,, 5	,, 3/10 ,,	,, 18 ,,
,,	,, 6	,, 1/10 ,,	,, 18 ,,

The death of the goat prevented further injections and tests being made. It will be seen that demonstrable antiendotoxins had developed in the goat and that the results were encouraging. After 12 injections of typhoid cell juice, 1/50 c.c. of the goat's serum protected a rabbit against 10 lethal doses of the typhoid endotoxin. This property was not present in 3 c.c. of normal goat's serum. It is not my intention in this paper to refer in detail to the production of agglutinins and bacteriolysins. It will be sufficient to state that whilst the amount of agglutinins present in the serum varied, the highest titrate obtained was in a dilution of 1/1,000,000. After 12 injections of typhoid cell juice, 1/10000 c.c. of the goat's serum protected guinea-pigs on intraperitoneal injection against 10 lethal doses of the *B. typhosus*. The serum was, therefore, proved to possess marked anti-endotoxic, agglutinative and bacteriolytic properties.

The next step was to control these results by the immunisation of fresh

goats, and to carry out the process in a still more careful manner. The method adopted is given in the following schedule :—

Nanny Goat II.—Received following intravenous injections of toxic cell juices of *B. typhosus*—

1905.				1905.			
Oct. 13	.....	1/20 c.c.	Ill.	Dec. 22	.....	1/8 c.c.	No symptoms.
„ 20	.....	1/20 „	No symptoms.	„ 29	.....	1/6 „	Ill.
„ 27	.....	1/15 „	Ill.	1906.			
Nov. 3	.....	1/15 „	No symptoms.	Jan. 5	.....	1/3 „	No symptoms.
„ 10	.....	1/10 „	No symptoms.	„ 12	.....	1/6 „	No symptoms.
„ 17	.....	1/10 „	Ill.	„ 19	.....	1/4 „	No symptoms.
„ 24	.....	1/10 „	Ill.	„ 26	.....	1/4 „	No symptoms.
Dec. 1	.....	1/10 „	No symptoms.	Feb. 2	.....	1/3 „	Ill.
„ 8	.....	1/8 „	Ill.	„ 9	.....	1/3 „	No symptoms.
„ 15	.....	1/8 „	No symptoms.				

III.—Tests of Serum of Goat II.

Date.	Test animal.	Amounts injected.	Results.
December 29	Rabbit 1	1/20 c.c. serum + 1 c.c. toxin.	Alive.
„	„ 2	1/50 „ + 1 „	Dead.
„	„ 3	1/100 „ + 1 „	Alive.
„	„ 4	Control, 1/5 c.c. toxin.	Dead, 2 hours.
„	„ 5	„ 1/10 „	Diarrhœa and collapse.
January 19, 1906...	„ 1	1/50 c.c. serum + 1 c.c. toxin.	Alive.
„ ...	„ 2	1/100 „ + 1 „	Dead.
„ ...	„ 3	Control, 1/10 c.c. toxin.	„
January 26	„ 1	1/50 c.c. serum + 1 c.c. toxin.	Alive.
„	„ 2	1/100 „ + 1 „	Dead.
„	„ 3	Control, 1/10 c.c. toxin.	„
February 2	„ 1	1/10 c.c. serum + 1 c.c. toxin.	Alive.
„	„ 2	1/20 „ + 1 „	„
„	„ 3	1/50 „ + 1 „	„
„	„ 4	1/50 „ + 1/2 „	„
„	„ 5	Control, 1/10 c.c. toxin.	Dead, 2½ hours.
„	„ 6	„ 1/20 „	„ 2 „
„	„ 7	„ 1/30 „	„ 18 „
February 9	„ 1	1/50 c.c. serum + 1 c.c. toxin.	Alive.
„	„ 2	1/50 „ + 1 „	„
„	„ 3	1/100 „ + 1 „	Dead.
„	„ 4	1/100 „ + 1/2 „	Alive.
„	„ 5	Control, 1/10 c.c. toxin.	Dead.
„	„ 6	„ 1/20 „	Diarrhœa and collapse.

Three goats are in process of immunisation by this method of small and gentle dosage and have not up to the present succumbed. It will be observed that whilst in the case of Goat I the dosage was raised from

1/20 to $2\frac{1}{2}$ c.c., the dose reached in Goat II when the final test was made only amounted to 1/3 c.c. of juices containing 10 to 12 milligrammes of solid matter. The figures demonstrate the production in considerable amount of an antiendotoxin. The highest titrate obtained was a neutralisation of 30 ascertained lethal doses of endotoxin by 1/50 c.c. of the serum of Goat II. The results confirmed those obtained in the case of Goat I, and were equivalent despite the injection of a much smaller gross amount of typhoid cell juice. The raising of the antiendotoxic value of the serum had likewise been accomplished without any serious disturbance in the health of the animal. This was the difficulty which had retarded the progress of the initial experiments.

Some tests were made as to the action of the serum on the endotoxin when each was injected separately. The results were as follows:—

Rabbit I.—Fifteen lethal doses of typhoid cell juice were injected into a vein of the right ear and 1 c.c. serum into a vein of the left ear. A second injection of 1 c.c. serum was given 20 minutes later. The animal survived.

Rabbit II.—Received five lethal doses of the toxic juice in the right ear and 1 c.c. serum in the left ear. The animal survived.

Rabbit III.—Received five lethal doses intravenous and $\frac{3}{4}$ hour later at the onset of toxic systems 2 c.c. of serum. The rabbit survived. The serum, therefore, acted on separate injection into the blood stream.

The serum of Goat II was also tested against the endotoxin of the cholera organism. One-half cubic centimetre of typhoid serum was added to three lethal doses of cholera cell juice, and the mixture, after incubation for 30 minutes at blood heat, was injected into a rabbit. The animal died $2\frac{1}{2}$ hours after the injection. One-half cubic centimetre of a typhoid serum, which had been found to protect against 30 lethal doses of typhoid endotoxin, did not protect a rabbit against three doses of cholera endotoxin, and was to this extent specific. The agglutinative power of the serum rose to 1/1,000,000, and 1/10000 c.c. protected the guinea-pig against 10 lethal doses of the typhoid bacillus.

The serum was finally tested in dilutions of 1/10, 1/100, and 1/500 c.c. for any evidence of a precipitin reaction on the fresh typhoid cell juices. The result was negative. There had been no appreciable development of precipitins in a serum containing at the time, when it was tested, marked antiendotoxic properties.

Conclusions.

1. The intravenous injection of the goat with the toxic cell juices of the *B. typhosus* (obtained under the conditions described) in small and carefully regulated doses resulted in the production of an antiendotoxin.

2. The antiendotoxic value, as so far tested, reached a point at which 1/50 c.c. of the serum neutralised 30 lethal doses of the toxic typhoid cell juice. This action was not demonstrable in 3 c.c. of normal goat's serum, and was obtained after about four months' treatment of the goat. The results, after a more rapid method of immunisation, are better *qua* goat and rabbit than those obtained by Dr. Besredka in the course of two years with dead and living bacilli *qua* horse and guinea-pig.

3. The serum was also agglutinative for the *B. typhosus*, the titrate rising to 1/1,000,000.

4. The serum was also bacteriolytic, 1/10000 c.c. neutralising 10 lethal doses of the *B. typhosus*.

5. The serum did not give a precipitin reaction with typhoid cell juices.

6. The serum whilst neutralising the typhoid did not neutralise the cholera endotoxin.

My next step will be to test in how far it is possible to obtain analogous results in the horse.

Analogous results have been obtained indicating the production of an antibody for the endotoxin of the cholera organism.

I am greatly indebted to Mr. E. T. Thompson for invaluable aid as well as for an important modification which has rendered the grinding process void of danger.

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NOTE ON THE OCCURRENCE OF DIPHTHERIA BACILLI IN MILK.

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Though milk has often been considered the medium by means of which outbreaks of diphtheria have been propagated, the diphtheria bacillus has only on rare occasions been isolated from the milk in a virulent form.

Bowhill (1899), Eyre (1899), Klein (1901), and Dean and Todd (1901) are at present the only authors who instance cases and there seems to be no reference to similar cases apart from English literature. In June of this year there was received at this Institute for bacteriological examination a sample of milk, supposed to be associated with an outbreak of diphtheria, from which the diphtheria bacillus was isolated in a virulent form. The outbreak consisted of two cases of diphtheria, with an interval of about six months between them, which occurred in a private house with a private dairy attached. After milking, the milk was conveyed to the milk-house, which is situated in the basement of the house, poured into a sterilised bottle and forwarded to the Institute.

Four guinea-pigs were inoculated subcutaneously with the centrifugalised deposit of the sample, two being inoculated on the 12th June 1906 and two on the 13th. On the morning of the 16th these four guinea-pigs were all dead. The post-mortem appearances presented by these guinea-pigs were characteristic of diphtheria. Subcultures were made on inspissated blood-serum from the local lesions at the site of inoculation and in guinea-pigs Nos. I and III a bacillus morphologically resembling the Klebs-Löffler Bacillus of Diphtheria was found and isolated on agar plates.

The virulence and specificity of these two strains, Nos. I and III, were tested as follows:

No. I.

		Result
Guinea-pig No. 1 received subcutaneously ½ c.c. of a 72 hours broth culture		Dead within 72 hours.
,, ,, 2 ,, ,, 1 ,, ,, ,, ,,		,, ,, ,,
,, ,, 3 ,, ,, 3 ,, ,, ,, ,,		,, ,, ,,
,, ,, 4 ,, ,, 3 ,, ,, ,, ,,	+ 1 c.c. Diphtheria Antitoxin (*L.I.P.M.*)	Alive and well.
,, ,, 5 ,, ,, 3 ,, ,, ,, ,,	+ 1 c.c. ,, ,, ,,	,, ,,

No. III.

,, ,, 6 ,, ,, ½ ,, ,, ,, ,,		Dead within 72 hours.
,, ,, 7 ,, ,, 1 ,, ,, ,, ,,		,, ,, ,,
,, ,, 8 ,, ,, 3 ,, ,, ,, ,,		,, ,, ,,
,, ,, 9 ,, ,, 3 ,, ,, ,, ,,	+ 1 c.c. Diphtheria Antitoxin (*L.I.P.M.*)	Alive and well.
,, ,, 10 ,, ,, 3 ,, ,, ,, ,,	+ 2 c.c. ,, ,, ,,	,,

In all the guinea-pigs which died from inoculation the post-mortem appearances were typical of diphtheria infection and in guinea-pigs Nos. 2, 3, 7 and 8 the bacillus of diphtheria was again isolated from the tissues.

This seems conclusive proof that the diphtheria bacilli were present in the milk in a virulent form. As to the source of the bacilli and their mode of entrance into the milk one is unable in the present case to come to a definite conclusion. Dean and Todd, and more recently Ashby, have described outbreaks of diphtheria, in the latter instance consisting of 75 cases. In both outbreaks ulceration of the teats of the cows supplying the milk was associated with the presence of virulent bacilli on the lesions, so that it seemed important to enquire into the condition of the udders and teats of the cows supplying the milk in this instance. I had the privilege, along with a veterinary surgeon, of inspecting the dairy and all the animals connected with it.

In all the cows the teats and udders appeared perfectly healthy and only in one cow did we find anything resembling what may have been the scar of a previous ulcer. Swabs taken from the throats of the milkman and dairymaid afforded negative results upon examination.

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UPON THE BACTERIOLOGY OF THE SUMMER DIARRHOEA OF INFANTS.

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Introduction.

The diarrhoeal diseases of infancy, on account of their appalling mortality, demand the most careful and thorough investigation.

A large number of inquiries have been made in various directions with reference to the predisposing and etiological factors in these diseases, for example, the influence of seasonal and hygienic conditions, of natural and artificial feeding, of milk pasteurization, etc.

These inquiries have yielded valuable results, to which it is beyond the scope of this paper to refer. Whilst recognizing that in infantile diarrhoeas many possible factors have to be taken into consideration, there exists a particularly virulent type occurring in summer, which there is every reason to believe has an infectious origin. To this type of infantile diarrhoea bacteriological research has been especially directed. Escherich[1] in Germany and Booker[2] in the United States were among the earlier observers to study the bacteriological flora of the diseased intestine. They were, however, unable to obtain evidence as to the presence of pathogenic organisms of a specific type in the diarrhoeic stools.

A fresh impulse was given to research by Shiga's[3] studies of Japanese dysentery in 1898, and the isolation by him of the *B. dysenteriae.* Shiga's results were confirmed by Flexner[4] in Manila in 1900, and by Kruse[5] in Germany in 1901. Acute bacillary or epidemic dysentery is now regarded as due to the bacillus first isolated by Shiga, with which the type described by Kruse is identical. The Flexner type of *B. dysenteriae* differs in certain cultural characteristics, and in its agglutinative properties from the former. A number of intermediate forms allied either to the Shiga-Kruse or the Flexner type of organism have since been described.

Bacilli of the dysentery group are widely distributed, and have been detected in cases of acute endemic, epidemic, sporadic, and institutional dysentery.

Duval and Bassett[6] in 1902 concluded that the dysentery bacillus "is an important, if not the most important, cause of the summer diarrhoeas of children." In 1903, the Rockefeller Institute for Medical Research undertook, under Dr. Flexner's direction, an investigation on the local or general occurrence of the dysentery bacillus in the summer diarrhoeas of children in the United States. The reports on the bacteriology of the cases are published in *Studies from the Rockefeller Institute*, vols. i and ii, 1904. I may quote from Dr. Flexner's summing up of the results.

> The type of *B. dysenteriae* which preponderated in the children is the so-called "Flexner-Harris" organism. The "Shiga" type of the organism is exceptionally met with, and occasionally both types are found in association. . . . Types of *B. dysenteriae* of less well-established properties have also been encountered. . . . The central fact brought out by this collective investigation is the frequent occurrence in the diarrhoeal diseases of children of a specific micro-organism, which hitherto has been held to be of specific pathogenic action in human beings, and to be the cause of that form of dysentery among adults and also among children which is characterized by necrotic and pseudo-membranous lesions of the intestine and marked infectiousness.

More recent papers are those by Dunn,[7] Parks, Collins, and Goodwin.[8] Critical observations on the above results are made in the papers of Weaver and Tunnicliffe[9] and of K. Collins.[10] Charlton and Jehle[11] consider that summer diarrhoea in children may be caused by various forms of micro-organisms of a non-dysenteric type, more particularly by the *B. coli communis*. They note the occurrence of "vast numbers" of streptococci in the green stools. Streptococci in large numbers were also found by the Rockefeller observers associated with the *B. dysenteriae*.

It remains to be determined how far the summer diarrhoeas of other countries conform in type to those of the United States.

Scope of Research.

Last summer (1905) I undertook the bacteriological examination of cases of acute infantile diarrhoea occurring in London. The results are sufficiently advanced to be made the subject of a preliminary report.

The clinical aspect of the disease here is different from that described in America. Blood in the stools is quite the exception, whereas in America it is apparently very frequent. There seems very little doubt, therefore, that we are not dealing with the same type of disease in the two countries, and that the causal agent may very well be different. This being the case, I determined to make a research into the bacteriology of as many cases of the disease in this country as I could collect during the time of its prevalence last summer. To this end it seemed advisable to collect all the aërobic bacteria of intestinal type present in the stools and intestines of these cases, excluding, however, all the lactose-fermenting and the gelatine-liquefying bacteria, in order to determine which

types were the most prevalent. This was controlled by an examination of the stools of healthy children of similar age, so that one might exclude from the list of possible causes any bacteria found constantly present in normal intestines. I am indebted to the staff of the Victoria Hospital for Sick Children, Chelsea, and also to that of the Hospital for Sick Children, Great Ormond Street, for their aid and co-operation in this matter, the former sending me the stools from 9 cases of infantile summer diarrhoea, and the latter either the stools or the intestines, or both, from 49 cases of that disease, as well as blood samples from each patient when obtainable, for agglutination reactions.

Of the 58 cases examined, 28 were diagnosed clinically as "acute infective diarrhoea," the stools from these being of the typical liquid consistency, green colour, and without blood. The remaining 30 cases were diagnosed as "catarrhal diarrhoea," blood being found in the stools of 4 of them; some of these latter were liquid and green, and of very similar appearance to those from the former cases.

Methods Adopted for the Isolation of the Bacteria.

A small portion of the material, either the faeces or scrapings from the mucous surface of the large or small intestine, was transferred to a tube of sterile peptone beef broth and an emulsion made; from this the bile-salt-neutral-red-lactose-agar-plates of MacConkey[12] were inoculated, and incubated for twenty-four hours at 37° C. On the following day all the colourless colonies (that is, non-lactose fermenters) were picked off and put into tubes of lactose broth. These tubes were then incubated for three days at 37° C., at the end of which time all those that had not produced acid and gas were retained and the rest discarded. The former were then used to inoculate gelatine tubes, and set aside for future investigation.

In the numerous instances where no colourless colonies were to be found on the agar plates, the same material, which had been kept frozen in the cold room, was used for reinoculating fresh agar plates, until colourless colonies had been obtained.

The advantages of the bile salt lactose agar were found to be very great in this research, as practically all except intestinal bacteria are excluded by the bile salt, and the lactose fermenters are readily separated out by means of the deep red colour produced by the fermentation of the lactose in the presence of neutral red; at the same time it was found advisable to apply the further test of growing the cultures from the colourless colonies on lactose broth for three days at 37° C., to exclude those bacteria that ferment lactose slowly.

The next step was to examine all the gelatine cultures that had not liquefied at the end of six weeks, and to grow them on the various media used for identification—namely, broths containing glucose, mannite, dulcite,

lactose and cane sugar, and litmus milk; to examine their morphology, motility, etc., and their capability of producing indol in peptone beef broth when incubated for five days. It was found when the gelatine liquefiers had been discarded, that the number of cultures to be examined amounted to 304, and each of these cultures was then grown on all the media above mentioned for identification.

Each bacillus isolated was, immediately after isolation, tested as to its agglutination properties with the blood of the patient from which it had been obtained, and the same blood was also tested against Shiga, Flexner, typhoid, Gaertner, paratyphoid A, and paratyphoid B organisms. The strength of the dilution of serum employed in each case was 1 in 30, and of the forty-four bloods tested only one gave an agglutination reaction with the bacillus isolated from its own patient, and two with one or more of the before-mentioned test bacilli. The result is unlike what was found in America, where the agglutination of the patient's blood with dysentery bacilli was so frequently demonstrated. This procedure involved great labour, and was most disappointing in its results.

Table I shows the results of the examination of the morphology and cultural characteristics of 67 organisms of the lactose non-fermenting class isolated from the intestines or faeces of patients suffering from infantile diarrhoea.

It will be seen on comparing the two tables that the cultural reactions of none of the bacteria found by me in infantile diarrhoea correspond with those of known pathogenic bacteria, with the exception of Nos. 6 and 7, which are similar as regards their reactions to *B. enteritidis* of Gaertner and *B. paratyphoid* B of Schottmüller respectively. That they are identical with these organisms is open to doubt, as they did not agglutinate when tested with specific serums even in such low dilutions as 1 in 20.

There is a partial resemblance between Nos. 3 and 4 and the Philippine dysentery bacillus of Flexner; No. 3, however, differs in its reaction on sorbite, on which it produces acid, whereas Flexner's bacillus causes no reaction, and No. 4 does not produce indol, whereas Flexner's bacillus produces an abundant indol reaction. It will be observed that No. 3 also resembles *B. typhosus* in that it produces acid on glucose, mannite, and sorbite; in fact, it has some of the characters of both Flexner's bacillus and the typhoid bacillus, differing from the latter in its absences of motility, its alkaline reaction on milk, and its indol production. It was an interesting fact that No. 3 agglutinated equally well with either a Flexner or typhoid serum, as if it were a connecting link between the two bacilli. No. 4 agglutinates well with Flexner serum, but scarcely at all with typhoid serum.

That No. 3 was an infective agent in the case of one patient is possible, as it was agglutinated in a 1 in 30 dilution of the patient's blood serum, whilst the same serum agglutinated typhoid bacilli in a similar dilution.

No. 5 resembles *B. dysenteriae* of Shiga in its cultural reactions, but differs in its distinct motility and its absence of agglutination with either a Shiga or Kruse serum. Nos. 10 to 14 are organisms found fairly commonly in drinking water, and for that reason seem to be of small importance.

No. 1, if one can argue from its prevalence, seems to be the most important organism which I have so far isolated, having been found in 28 cases out of the 58 examined, and in 17 of these it was the only lactose non-fermenting organism present. It resembles in some respects the bacillus of hog cholera of McFadyean, differing, however, in its alkaline reaction on litmus milk, its greater production of indol, and in its failure to produce acid and gas on arabinose, maltose, and dextrin. Bacillus No. 1 produces indol in glucose broth in the presence of an excess of glucose, differing in this respect—as Dr. A. Harden kindly demonstrated for me—from most of the other indol-producing bacteria. That it produces true indol and not skatol-carboxylic acid I proved by distillation.

To determine in how far all the 28 cultures of No. 1 were identical each was grown on 11 additional media, as also was the bacillus of hog cholera of McFadyean, which in some respects resembles them. The cultures of Nos. 3 and 4 were also compared in the same way with all obtainable cultures of dysentery of the Flexner type, including two cultures isolated from infantile diarrhoea in America (Duval, Baltimore, and Duval, New York). Various strains of *B. dysenteriae* (Shiga and Kruse) were also tested in the same way, as also *B. typhosus*. To these were added three strains of dysentery bacilli isolated by Eyre from asylum dysentery, and two strains of bacilli isolated by MacConkey from monkey's faeces, one of which resembled *B. dysenteriae* (Flexner), and the other *B. dysenteriae* (Shiga).

In compiling Table II, two laboratory strains of *B. dysenteriae* (Flexner, Philippines) were used, and one from Kral; they were found to agree in all their reactions. In the same manner, three strains of *B. dysenteriae* (Shiga) were tested, and agreed culturally in every respect —they were obtained from Berlin, Kral, and the Institute. Two strains of *B. dysenteriae* (Kruse), one from Laar, and one from Kral were also found to be identical. It will be noticed that no cultural differences were found between *B. dysenteriae* (Shiga) and *B. dysenteriae* (Kruse), which many observers now regard as being identical.

The long list of media used proved very useful in distinguishing between bacilli Nos. 1, 3, and 4, and the other known bacteria which resemble them in some respects. It will be noted that No. 1 is quite different from the bacillus of hog cholera (McFadyean), and that Nos. 3 and 4, although of the dysentery type, are not identical with any known dysentery bacilli, nor with either of the Duval bacilli isolated from infantile diarrhoea in America.

No. 1, which will be subsequently shown to be of pathogenic importance, is a motile rod, slightly smaller than

TABLE I.—*Morphological and Cultural Tests applied to the various Bacteria Isolated.*

No.	No. of Cases in which Found.	Morphology.	Glucose.	Mannite.	Dulcite.	Lactose.	Cane Sugar.	Litmus Milk.			Litmus Whey.		Indol.	Sorbite.
								1 Day.	3 Days.	15 Days.	1 Day.	7 Days.		
1	28	B. Motile	A. G.	—	—	—	—	0	0	Alk.	Alks.	Alks.	+ +	
2	3	B. Non-motile	A. G.	—	—	—	—	A.	A.	Alks.	A.	Alk.	—	
3	5	,,	A.	A.	—	—	—	A.	A.	0	A.	A.	+ +	A.
4	2	,,	A.	A.	—	—	—	A.	A.	0	A.	A.	—	—
5	4	B. Motile	A.	—	—	—	—	A.	0	Alk.	A.	Alk.	—	
6	1	,,	A. G.	A. G.	A. G.	—	—	A.	A. S.	Alk.	A.	Alk.	—	
7	2	,,	A. G.	A. G.	A. G.	—	—	A. S.	A.	Alk.	A.	Alk.	+	
8	1	B. Non-motile	A. G.	A. G.	A. G.	—	—	A.	0	A.	A.	A.	—	
9	1	,,	A. G.	A. G.	A. G.	—	—	A.	A.	Alk.	A.	Alk.	—	
10	4	,,	A. G.	A. G.	A. G.	—	A. G.	A.	A.	A. C.	A.	A.	+ +	
11	3	B. Motile	A. G.	A. G.	A. G.	—	A. G.	A.	Alk.	Alk.	A.	Alk.	+	
12	2	,,	A. G.	A. G.	—	—	A. G.	A.	A.	A. C.	A.	A.	—	
13	1	,,	A. G.	A. G.	—	—	A. G.	A.	A.	A. C.	A.	A.	+	
14	5	,,	A. G.	A. G.	—	—	—	A.	A.	Alks.	A.	Alks.	+ +	
15	2	Streptococci	A.	A.	A.	A.	A.	A. S.	A.	A.	A.	A.	—	
16	1	,,	A.	—	—	A.	A.	A.	A. C.	A. C.	A.	A.	—	
17	1	Coccus	A.	—	—	—	—	0	A.	A. C.	0	A.	—	
18	1	,,	A. G.	A. G.	—	—	—	A.	0	Alk.	A.	Alk.	+	

In order to compare the above table with the morphology and cultural reactions of some of the best known pathogenic intestinal bacteria, I add the following table compiled from my previous work. (Reference: Some Observations upon the Micro-organisms of Meat Poisoning, and their Allies, BRITISH MEDICAL JOURNAL, June 10th, 1905, p. 1259.)

Bacterium.	Morph	Glucose.	Mannite.	Dulcite.	Lactose.	Cane Sugar.	Litmus Milk.			Litmus Whey.		Indol.	Sorbite.
							1 Day.	3 Days	15 Days.	1 Day.	7 Days.		
B. dysentery, Flexner, Philippines	B. Non-motile*	A.	A.	—	—	—	A.	Alks.	Alks.	A.	Alks.	+	
B. dysentery, Shiga ...	,,	A.	—	—	—	—	A	Alks	Alks.	A.	Alks.	—	
B. typhoid	B. Motile	A.	A.	—	—	—	A.	A.	A.	A.	A.	—	A.
B. enteritidis, Gaertner...	,,	A. G	A. G.	A. G.	—	—	A.	Alk.	Alk.	A.	Alk.	—	
B. paratyphoid B, Schottmüller	,,	A. G.	A. G.	A. G.	—	—	A.	Alk.	Alk	A.	Alk.	+ Slight	
B. paratyphoid A, Schottmüller	,,	A. G.	A. G.	A. G.	—	—	A.	A.	A.	A.	A.	+	
B. hog cholera, McFadyean	,,	A. G.	—	—	—	—	A.	A.	A.	A	A.	+ Slight	

A. = Acid. G. = Gas. C. = Clot. S. = Slight. — = No reaction.

the typhoid bacillus, and, like the latter, multi-flagellated. As far as I can ascertain, this bacillus has not been recognized before. Dr. Houston very kindly examined his notes for me on the numerous bacteriological analyses of human faeces, sewage, and water, made by him, but was unable to find any record of a bacillus of this type, nor at the Lister Institute has this bacillus been isolated from the numerous samples of drinking water sent for analysis. This fact led me to make a bacteriological examination of the stools of twenty normal children under 2 years of age admitted to the Hospital for Sick Children, Great Ormond Street, for such operations as harelip or cleft palate.

The method of examination was identical with that employed by me in the cases of infantile diarrhoea, with the result that out of the 20 cases only one gave a colony at all resembling No. 1, and none gave colonies resembling Nos. 3 and 4. The culture resembling the No. 1 differed from those isolated from the cases of children's diarrhoea in its failure to produce alkalinity in litmus milk, in which it produced no change of reaction, and its production of a smaller amount of indol in peptone beef broth. This bacillus I subsequently found to be non-pathogenic for experimental animals, whereas No. 1 is distinctly pathogenic for young rabbits and rats when administered by the mouth.

The examination of the stools of normal infants established the fact that the non-lactose fermenting bacteria are very infrequent in the normal faeces, as, in spite of repeated plating on lactose agar, it was very difficult to obtain any colourless colonies. In addition to the single colony resembling the No. 1 beforementioned, I was only able to isolate one colony of a bacillus resembling No. 5, and a few colonies resembling Nos. 10 and 14.

Agglutination Reactions with the Blood of Patients suffering from Infantile Diarrhoea.

Forty-four samples of blood were tested against all the bacilli isolated from the patients, against the dysentery bacilli of Flexner and Shiga, and the typhoid, Gaertner, paratyphoid A, and paratyphoid B organisms, in each case in a dilution of 1 in 30. Only one, however, gave a reaction with the bacillus isolated from its own patient, namely, bacillus No. 3, which was agglutinated by the patient's serum.

In the case of two of the patients, the serum agglutinated known bacilli, one in a case in which bacillus No. 1 was present in pure culture (lactose fermenters excepted), the serum agglutinating both typhoid and Gaertner bacilli, in a dilution of 1 in 60. The other case was that of the serum of a patient from whom bacillus No. 1 was isolated; this serum gave a reaction with the typhoid bacillus in a dilution of 1 in 30.

The time that had elapsed between the onset of the disease and the obtaining of the patient's blood was found

TABLE II.—*The various cultures are so arranged that those isolated by me from*
resemble

Bacterium.	Motility.	Glucose.	Levulose.	Mannite.	Dulcite.	Maltose.	
No. 1	B. Motile	A. G. S.	A. G. S.	—	—	—	
B. hog cholera, McFadyean...	,,	A. G. S.	A. G. S.	—	—	A. G. S.	A.
No. 3	B. Non-motile	A.	A.	A.	—	A.	
No. 4	,,	A.	A.	A.	—	—	
B. dysentery, Flexner, Philippines ...	,,	A.	A.	A.	—	A.	
B. dysentery, Gray	,,	A.	A.	A.	—	A.	
B. dysentery, Strong	,,	A.	A.	A.	—	A.	
B. from infantile diarrhoea, Duval, Baltimore	,,	A.	A.	A.	—	A.	
B. from infantile diarrhoea, Duval, New York	,,	A.	A.	A.	—	A.	
B. from monkey's faeces, MacConkey II..	,,	A.	A.	A.	—	A. S.	
B. typhoid	B. Motile	A.	A.	A.	—	A.	
B. dysentery, Shiga	B. Non-motile	A.	A.	—	—	—	A
B. dysentery, Kruse	,,	A.	A.	—	—	—	A
B. asylum dysentery, Eyre 7	,,	A.	A.	—	—	—	A
B. asylum dysentery, Eyre 8	,,	A.	A.	—	—	—	
B. asylum dysentery, Eyre 9	,,	A.	A.	—	—	A. S.	A
B. from monkey's faeces, MacConkey I ..	,,	A.	A.	—	—	—	

A. = Acid. G. S. =
G. = Gas. Alk. =

ile diarrhoea can be easily compared with known bacteria which, in some respects,

Cane Sugar.	Lactose.	Galactose.	Inulin.	Amygdalin.	Salicin.	Arabinose.	Raffinose.	Sorbite.	Erythrite.	Indol.	Litmus Milk.		
											1 day.	3 days.	15 days.
—	—	A. G. S.	—	—	—	—	—	—	—	+ +	0	0	Alk.
—	—	A. G.	—	—	—	A. G	—	—	—	+ Slt.	A.	A.	A.
—	—	A.	—	—	—	A.	—	A.	—	+ +	A.	A.	0
—	—	A.	—	—	—	A.	A. S.	—	—	—	A. S.	0	Alks.
—	—	A.	—	—	—	A.	A. S.	—	—	+	A.	0	Alks.
—	—	A.	—	—	—	A.	A. S.	—	—	+	A.	0	Alks.
—	—	A.	—	—	—	A.	A. S.	—	—	+	A.	0	Alks.
—	—	A.	—	—	—	A.	A. S.	—	A. S.	+	A.	0	Alks.
—	—	A.	—	—	—	A.	A. S.	—	A. S.	+	A.	0	Alks.
—	—	A.	—	—	—	A. S.	A. S.	—	A. S.	+ trace	A.	0	Alks.
—	—	A.	—	—	—	—	—	A.	—	—	A.	A.	A.
—	—	A.	—	—	—	—	—	—	A. S.	—	A.	0	Alks.
—	—	A.	—	—	—	—	—	—	A. S.	—	A.	0	Alks.
—	—	A.	—	—	—	—	—	—	A. S.	—	A.	0	Alks.
—	—	A. S.	—	—	—	A. S.	—	—	—	—	0	0	0
—	—	A.	—	—	—	—	—	—	—	—	A.	0	Alks.
—	—	A.	—	—	A.	A. S.	—	—	A.	—	A.	0	Alks.

Gas.
nity.

Alks. = Slight Alkalinity.
— = No Reaction.

to have varied from twenty-four hours to two months, practically all intermediate periods being represented. Of the three instances in which a reaction was obtained, one was with that of a blood taken at the end of twenty-four hours after the onset, one at the end of fifteen days, and the third at the end of two months.

The other agglutination reactions investigated were those relating to the bacilli found by me with known serums. In these experiments bacillus No. 3 was found to agglutinate equally well with both dysentery (Flexner) serum, and typhoid serum, thus accentuating its resemblance to these organisms. No. 4 was found to agglutinate well with dysentery (Flexner) serum, but only feebly and in low dilutions, with typhoid serum. No. 5, which but for its motility culturally resembles the *B. dysenteriae* (Shiga), failed to agglutinate with Shiga serum even in so low a dilution as 1 in 20. No. 7, which culturally resembles *B. paratyphoid* B of Schottmüller, failed to agglutinate with a paratyphoid B serum.

The various cultures of bacillus No. 1 obtained from different patients did not in every instance interagglutinate. Five rabbits were immunized with five different strains of this bacillus, and, although some did, there were many that failed to agglutinate with any of the five serums.

All the cultures, however, obtained from the same patient were found to agglutinate with a serum prepared by immunizing a rabbit with one of their number. The bacillus No. 1 in this respect forms no exception to other groups of bacteria, such as the colon and Gaertner groups, the members of which do not invariably interagglutinate.

The Pathogenicity of the Bacilli Isolated from the Patients.

For this test, organisms Nos. 1, 3, and 4 were selected, as they appeared to be the most important.

Two young rats were fed with half an agar culture each of No. 3 mixed with their food; on the following day both rats were dead, and the bacillus was recovered from the spleen of one of them. There was no evidence of diarrhoea having been caused in either case. Two young rats were also fed with No. 4 in the same way; one rat died on the following day, and the other on the second day. The bacillus was not recovered from either of these rats, though it was looked for in the spleen and in the blood. There was no diarrhoea before death.

Eighteen successful feeding experiments were made with the No. 1 bacillus, twelve with young rats and six with young rabbits. Half an agar tube proved to be invariably fatal in the case of young rats, and an agar tube in the case of young rabbits. Death generally took place within twenty-four hours, and was preceded by violent diarrhoea. The bacilli were in each case recovered from the spleen in pure cultures, and in a few instances from the heart blood as well. That this was not a mere *post-mortem*

invasion was proved in one instance by killing the sick animal, and taking out the spleen immediately. The No. 1 bacillus was recovered in pure culture in this instance from the spleen.

As a control experiment, two young rats were fed on half an agar tube each of a mixture of three different varieties of *B. coli* freshly isolated from human faeces. One young rat died at the end of four days, and the other was still alive at the end of a month, proving that the virulence of No. 1 bacillus is very much higher than that of *B. coli.*

The first experiments to investigate the pathogenicity of the No. 1 bacillus were by intraperitoneal and intravenous injection. These, however, entirely failed to have any effect on the animals experimented upon, even so large a dose as 1 c.cm. of a very thick emulsion from an agar culture injected intravenously into a small rabbit failing to produce any result, nor did ¼ c.cm. injected intraperitoneally into a mouse or 1 c.cm. into a guinea-pig have any effect. No. 1 bacillus, therefore, seems only to be pathogenic when introduced directly into the digestive tract.

The pathogenicity was next investigated of the bacillus which had been isolated from the stools of a normal infant, and which resembled the No. 1 bacillus in all its cultural reactions with the exception of its reaction in litmus milk. An agar tube culture of this was mixed with the food of two young rats, this being the dose that proved invariably fatal in the case of the No. 1 bacillus. The young rats, however, were not in the least affected by it, and were still alive at the end of a month, proving conclusively that this bacillus differs widely in its pathogenicity from the No. 1 bacillus.

Toxin Formation.

The next point of investigation was to determine whether Nos. 1, 3, and 4 produced a toxin. For this purpose flasks of alkaline peptone beef broth were inoculated with each of these cultures, and incubated for four weeks at 37° C. At the end of this time they were filtered through a Berkefeld filter, and the filtrate was injected in doses of ½, 1, 2, and 5 c.cm. intravenously into rabbits.

The first experiment was made with No. 3 filtrate, which failed to produce any effect on a rabbit even when injected intravenously in so large a dose as 5 c.cm. The filtrate of No. 4 produced no effect in any dose less than 5 c.cm., when it caused death, preceded by diarrhoea; so that, although in the feeding experiments this bacillus caused no diarrhoea before death, its toxin when injected intravenously seems to have that effect.

The filtrate from the broth culture of No. 1 bacillus was found to cause death in about twelve hours when injected intravenously into a rabbit in a dose of 2 c.cm., in some instances causing diarrhoea before death. The toxin

developed seems to be somewhat unstable, as the same dose failed to produce any symptoms after having been kept in a cold room at a temperature of about 0° C. for a week. The toxicity of the filtrate was found to be no greater at the end of four weeks than it was at the end of one week, the minimum lethal dose being also 2 c.cm. It may be that we are here dealing, as in the case of dysentery, with toxins of an intracellular type.

Conclusions.

These results, together with the evidence drawn from other sources, indicate that the bacillus No. 1 is not an organism normally present in human stools, sewage, or in drinking water. From its prevalence in the stools and intestines of cases of infantile diarrhoea (in 28 cases out of 58), also from the fact that it causes death, preceded by diarrhoea in young animals, in the spleens of which it is invariably found in pure culture, one is led to conclude that this bacillus may possibly be a factor, or one of the factors, in the disease. A very significant fact is that the bacillus was isolated in pure culture (together with lactose-fermenting bacilli) from the stools of a nurse who had contracted diarrhoea from the patients in the ward set apart for this disease in the Hospital for Sick Children, Great Ormond Street.

The evidence in favour of bacillus No. 3 being either the cause, or a cause of the disease, is perhaps almost equally strong, for although it was isolated in only 5 cases out of 58, one of these cases points very strongly to its being an infective agent, from the fact that the patient's blood agglutinated this bacillus, and also the typhoid bacillus. It will be remembered that it was proved by experiment that this bacillus was agglutinated equally well by dysentery (Flexner) serum, and by typhoid serum. That this patient's serum, therefore, should agglutinate typhoid bacilli, as well as its own bacillus, is a very strong point in favour of this bacillus being infective in this particular patient at least. It will be remembered, too, that it proved pathogenic to young rats which were fed on it, although no diarrhoea was produced.

The evidence in favour of bacillus No. 4 being a factor in the disease is based principally on its resemblance to the Flexner group of dysentery bacilli, which have been assigned as the cause of the disease in America. This bacillus, too, proved pathogenic to young rats when they were fed on it.

Bacillus No. 1 and bacilli Nos. 3 and 4 were found with about the same relative frequency in the cases clinically diagnosed as "acute infective diarrhoea" and "catarrhal diarrhoea." Bacillus No. 1 was found in 11 out of the 28 cases of the former type, and in 17 out of the 30 cases of the latter type of the disease. Bacillus No. 3 was found in 2 cases of "acute infective diarrhoea" and in 3 cases of "catarrhal diarrhoea," and bacillus No. 4 in 1 of the former and 2 of the latter. It will thus be seen that

the relationship between the clinical diagnosis and the bacteriology of the disease is not well marked. Dr. Batten informs me in this respect that the distinction between these two types is not very definite, and it may very well be that the acute infective type is merely an aggravated form of the catarrhal.

The presence of a certain bacillus in the stools, or in the intestines, in cases of disease, even although that bacillus be not found in the stools of healthy individuals, hardly proves it to be the cause of the disease. If, however, it has been found to invade the blood or the spleen, this evidence is very strongly in its favour. Unfortunately, neither the blood nor the spleen of fatal cases were examined bacteriologically, so that the presence or absence of bacteria in these localities are matters for future investigation by me next summer.

My thanks are due to Dr. F. E. Batten, who took the greatest interest in this research, and supplied me with the material from the Hospital for Sick Children, Great Ormond Street; also to Dr. Allan Macfadyen, at whose suggestion and in whose laboratory the work was done, for his most valuable assistance and advice.

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ON THE OCCURRENCE OF TOXIC COMPOUNDS OF TETANUS TOXIN AND ANTITOXIN, TETANUS TOXIN AND BRAIN EMULSIONS[1].

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(*From the Lister Institute of Preventive Medicine.*)

Introduction.

Ehrlich had not long announced his theory of immunity, when Wassermann and Takaki (1898) published their discovery, that the brain matter of the guinea-pig was capable of neutralising tetanus toxin. This was taken as a demonstration of the existence of Ehrlich's receptors in the cells of the normal brain.

Wassermann's strongest argument for the chemical nature of the combination between tetanus toxin and brain matter is its specificity.

A certain parallelism has been demonstrated to exist between the susceptibility and the toxin-neutralising property of the brain of an animal. Thus the cerebral cortex of highly susceptible animals, man and horse (Wassermann) and mouse (Metchnikoff) is strongly antitoxic, the brain of the less susceptible, rabbit, pigeon and fowl (Wassermann and Metchnikoff), has a feebler action, whereas in the case of the highly refractory tortoise and frog, the brain has no neutralising power.

Wassermann and Takaki thought at first, that brain and toxin

[1] This research was carried out in the laboratory of the Serum Department of the Lister Institute. To the Bacteriologist-in-charge of that department, Dr Dean, I am deeply indebted for inspiration and kindly criticism.

combined in the proportions which give the neutral mixture, just as on Ehrlich's theory a neutral mixture of toxin and antitoxin is one in which the combining affinities of both substances are satisfied. Metchnikoff (1898), however, pointed out that it was not necessary to conclude that the toxin was neutralised by the brain emulsion in the same way as by antitoxin. Metchnikoff, Roux and Salimbeni (1896) had shown that leucocytes could destroy cholera toxin, which they ingest while it is still retained within the bodies of the vibrios. Vibrios and toxin together are digested by the phagocytes. On the other hand a corresponding dose of toxin extracted beforehand from the vibrios, kills the test animal rapidly. Metchnikoff therefore argues, that the toxin in Wassermann's experiment may be absorbed physically by the solid particles of brain, and in this state may be taken up and digested by phagocytes.

Besredka (1903), went into the matter more thoroughly and showed that whatever the nature of the reaction, brain matter can combine with very much more toxin than it can neutralise. Besredka saturated the brain matter with a large excess of toxin, and then washed the combination in a centrifuge six times, till the washings only produced slight tetanus in a mouse, in a dose of 20 drops. One drop of the sediment of this brain matter was however sufficient to produce rapidly fatal tetanus in a mouse. The neutral mixture does not therefore represent a saturated chemical compound.

From the above brief outline of the literature of the subject it is evident that the nature of the combination between tetanus toxin and brain substance, is still open to discussion.

The main problems with which we have to deal are whether this combination is specific and whether it belongs to the same order of phenomena as the combination between antitoxin and toxin.

It seemed possible that a minute study of the quantitative combining relations under different conditions between brain substance and antitoxin respectively with tetanus toxin might throw further light upon the nature of their interaction.

The present paper records such a comparative study of the reaction between antitoxin and toxin, with that between brain and toxin.

In comparing a brain emulsion with an antitoxic serum, we are not really comparing a solid with a solution. The brain particles contain water, and, chemically considered, are equivalent to an emulsion of a colloid. Antitoxin is associated with the globulin of serum, and W. B. Hardy (1905) has shown that the transition from solution to precipitate, in the case of globulins, is not to be considered as a change

of state. The colloidal particles in the "solution" contain water and are bounded by a surface, but are too small to cause interference with a beam of light, and are easily held in suspension in water. The particles of the "precipitate" contain less water, and are larger, and therefore are visible, and sink to the bottom of the test tube. The difference between the clear "solution" and the flocculent "precipitate" is one of degree, not of kind.

The actual conditions under which tetanus toxin is absorbed by brain emulsion and antitoxic serum respectively are not therefore so dissimilar as might be supposed at first sight, and as shown by Danysz (1902) and Bordet (1903) some of the phenomena of toxin-antitoxin union suggest that this action is of the nature of absorption rather than that of combination between a strong (Ehrlich) or a weak (Arrhenius and Madsen) acid and base.

The relation of toxin absorbed by brain substance to original toxin concentration.

It seemed probable that the amount of toxin fixed by an emulsion of brain, would be some function of the amount of toxin originally present. This is the case, as may be shown by placing brain matter in the presence of varying quantities of toxin, centrifuging and estimating the amount of toxin remaining free in the supernatant fluid. By subtracting the observed free toxin from the known quantity originally present, we deduce the amount which has been fixed by the brain.

The following notation is used in the account of experiments:

−, =, ≡, denote increasing degrees of severity of local tetanus.

×, general tetanus, the animal certainly about to die within a few hours. Such animals were killed.

+, found dead.

The toxin used was prepared in April 1906. It was the filtrate from a 10 days' growth of tetanus bacilli in beef bouillon, previously freed from sugar by the growth in it of colon bacilli. It was preserved in the dark, at 10° C. under toluol.

This toxin was tested on mice on May 8th and October 22nd 1906, with the result given in Table I. It will be seen that the toxin lost a little in the five months interval.

TABLE I.

Dose per gram weight	Result after days given					
May 8, 1906	1	2	3	4	5	6
·000005	0	–	≡	≡	≡	≡
·00001	0	=	≡	+		
·00002	0	≡	≡	+		
·00004	0	≡	+			
·0001	0	×				
Oct. 22, 1906						
·000015	0	–	=	+		
·00003	0	=	≡	+		
·00006	–	≡	+			
·00012	=	+				

Experiment I. October 15th. Six tubes were charged with increasing doses of toxin; saline was added to make up the volume to 4 c.c. To each tube was added 2 c.c. of a 20 per cent. emulsion of guinea-pig's brain. The mixtures were allowed to stand for 11 hours at 10° C. and then a sample was removed from each tube and centrifuged. The supernatant fluid was tested for toxicity with the result given in Table II. In this table one mouse is selected for each tube, from a series all of which had different doses.

TABLE II. *Experiment I.*

October 16, 1906.

Total toxin	Toxic units lethal for 1 gm. of mouse in each ·024 c.c.	Total brain	Dose of supernatant fluid per gm. weight of mouse	Date and result									Toxic units, found in each ·024 c.c. of supernatant fluid
				17th		18th		19th		20th	21st	22nd	
				M.	E.	M.	E.	M.	E.	M.	M.	M.	
·3 c.c.	20	·4 gm.	·048 c.c.	0	–	=	=	≡	≡	≡	≡	+	$\frac{1}{8}$
·6	40	·4	·024	0	0	=	≡	≡	≡	≡	≡	+	$\frac{1}{4}$
1·1	73	·4	·048	0	=	≡	≡	×					$\frac{1}{2}$
1·6	107	·4	·024	0	–	≡	≡	+					1
2·4	160	·4	·012	0	–	≡	≡	+					2
4	267	·4	·003	0	–	≡	≡	+					8

The relation existing between the toxin found free in solution, and that combined with the brain matter.

In my experiments I have chosen as the unit of volume, not the whole contents of the test tube, but ·024 c.c. because the test mice had a dose of this volume, or some multiple or whole fraction of it, for each gram body weight. The toxic unit, in which I have expressed the

quantities of toxin, is the amount per gram weight necessary to kill a mouse in 3 days (cf. Table I.). The unit of brain matter is the mass contained in ·024 c.c., that is ·0016 gram.

I have plotted the results in Fig. 1 where the ordinates represent the observed free toxin, and the abscissae the amount of toxin fixed by unit mass of brain matter. In the same figure I have plotted a curve calculated from Arrhenius and Madsen's equation for the reaction between toxin and antitoxin. It will be seen that the reaction between

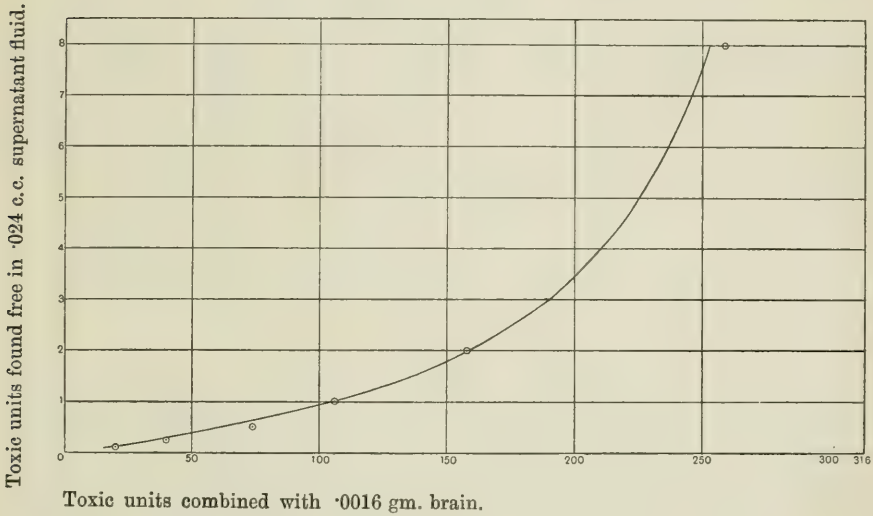

Fig. 1.

toxin and brain may very well follow the same law. The limit of error in the experiment is wide, so that I would not insist further on this agreement, but I hope to follow out this line of inquiry by a more accurate method.

The calculated and observed figures are given below:

Free toxin in ·024 c.c.	Observed combined toxin	Calculated combined toxin
1/8	20	18·6
1/4	40	35
1/2	72	63
1	106	105
2	158	158
8	259	253

The form of equation used was

$$\left(\frac{\text{Free toxin}}{\text{volume}}\right)\left(\frac{\text{Uncombined brain}}{\text{volume}}\right) = K\,\frac{\text{Combined brain toxin}}{\text{volume}}.$$

The volume being unity may be neglected.

The dissociation constant, K, was found to be 2, and the amount of toxin equivalent to unit mass of brain (·0016 gram) was found to be about 316 toxic units.

Now whether we accept Madsen's or Bordet's view of the reaction between toxin and antitoxin, toxic combinations between the two must exist, comparable to Besredka's "cerveau toxique." These combinations will be formed whenever a toxic mixture of toxin and antitoxin is prepared. Their recognition, however, is difficult, because the presence of free toxin is a necessary condition of their formation. In spite of this they have been isolated in the case of ricin and antiricin. The combinations of ricin and antiricin under favourable circumstances form a precipitate, and Danysz (1902) found that, when ricin was present in excess, the precipitate contained practically all the ricin. He concludes justly that toxin and antitoxin "impregnate each other in variable proportions."

One cannot separate the compound of tetanus toxin with its antitoxin by the centrifuge, but one can remove the free toxin by allowing it to combine with brain matter, and centrifuging. The combined toxin and antitoxin then remain in the solution. In this way I have isolated toxic combinations of tetanus toxin with antitoxin, exactly comparable to Besredka's "cerveau toxique" and the toxic precipitate of Danysz.

In this series of experiments an antitoxin was used of which ·0032 c.c. mixed with 1 c.c. of my toxin gave a slightly toxic mixture. This was tested at the concentration at which the experiments were to be carried out. The amounts given in Table III. were diluted so that the volume in each tube was 8 c.c.; and mice received ·024 c.c. per gram body weight.

To show the combination of antitoxin with an excess of toxin above that contained in the neutral mixture, I chose a dose of antitoxin (·00016 c.c.) which would neutralise ·04 c.c. of toxin.

Test tubes were charged with ascending doses of toxin, beginning in the first tube with the $L+$ dose for the above quantity of antitoxin.

The volume in each tube was made up to 4 c.c. with ·85 per cent.

salt solution. The antitoxin contained in 2 c.c. of the same salt solution was then added, and the mixtures were set at 10° C. for some hours (Ta in the tables). After this time, 2 c.c. of a brain emulsion was added to each tube, and the series set aside again for a time (Tb) which was allowed for the brain matter to combine with the free toxin present. Then a sample from each tube was centrifuged, and the clear fluid tested on a mouse.

TABLE III.

September 3, 1906.

Toxin	Antitoxin	Result and date						
		4	5	6	7	8	9	10
·1 c.c.	·00024 c.c.	0	≡	≡	+			
·1	·00028	0	≡	+				
·1	·00032	0	0	–	–	survived		
		October 4, 1906.						
		5	6	7	8	9	10	11
·7 c.c.	·0016 c.c.	–	+					
·6	·0016	0	≡	+				
·5	·0016	0	=	≡	+			
·4	·0016	0	0	0	0	–	=	

In each experiment a control series was set up, in which no antitoxin was used. The same doses of toxin were diluted to 6 c.c.; and 2 c.c. of the same brain emulsion were added to each tube, immediately after the brain had been added in the antitoxin series. Samples from the control series were centrifuged immediately after the similar samples from the antitoxin series.

The test dose per gram weight of mouse was ·024 c.c. in every case.

TABLE IV. *Experiment II.*

August 30, 1906.

Ta = 21 hrs. at 10° C. Tb = 35 mins. at 36° C.

Toxin	Antitoxin	Brain	Date and result							
			31	1	2	3	4	5	6	7
·05 c.c.	·00016 c.c.	·3 gm.	•	–	–	–	–	–	–	–
·1	·00016	·3	•	–	≡	≡	≡	≡	×	
·2	·00016	·3	•	=	≡	+				
			Control Series.							
·05	0	·3	•	0	0	0	–	–	0	0
·1	0	·3	•	0	–	–	–	–	0	0
·2	0	·3	•	–	≡	≡	≡	≡	≡	≡

TABLE V. *Experiment III.*

September 4, 1906.

Ta = 15½ hrs. at 10° C. Tb = ½ hr. at 36° C. and 2 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 5	6	7	8
·05 c.c.	·00016 c.c.	·2 gm.	0	0	0	0
·07	·00016	·2	0	0	0	–
·1	·00016	·2	0	0	–	≡
·14	·00016	·2	0	–	≡	≡
·2	·00016	·2	0	=	≡	+
		Control Series.				
·05	0	·2	0	0	0	0
·07	0	·2	0	0	0	0
·1	0	·2	0	0	0	0
·14	0	·2	0	0	–	–
·2	0	·2	0	0	–	–

TABLE VI. *Experiment IV.*

September 17, 1906.

Ta = 15 hrs. at 10° C. Tb = 6 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 18	19	20	21	22	23	24
·05 c.c.	·00016 c.c.	·2 gm.	0	0	0	0	0	0	0
·07	·00016	·2	0	–	=	≡	≡	≡	×
·1	·00016	·2	0	0	–	=	=	=	=
·14	·00016	·2	0	=	≡	≡	≡	≡	+
·2	·00016	·2	0	=	≡	≡	≡	+	
		Control Series.							
·05	0	·2	0	0	0	0	0	0	0
·07	0	·2	0	0	0	0	0	0	0
·1	0	·2	0	0	0	0	0	0	0
·14	0	·2	0	0	0	0	0	0	–
·2	0	·2	0	0	–	–	·	–	+

TABLE VII. *Experiment V.*

October 4, 1906.

Ta = 20 hrs. at 10° C. Tb = 5 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 5	6	7	8
1 c.c.	·0016 c.c.	·2 gm.	0	+		
2	·0016	·2	0	+		
4	·0016	·2	≡	+		
		Control Series.				
·5	0	·2	0	=	≡	+
1·5	0	·2	–	≡	+	
3·5	0	·2	=	+		

TABLE VIII. *Experiment VI.*

June 19, 1906.

	Toxin made up to ·5 c.c.	Antitoxin made up to 2·5 c.c.	Brain made up to 2·5 c.c.	Date and result 20	21	22	23	24	25
Ta = 0	·0625 c.c.	·0001 c.c.	·05 gm.	0	mouse lost				
	·25	·0001	·05	0	≡	≡	+		
Ta = 32′	·0625	·0001	·05	0	0	0	0	0	0
	·25	·0001	·05	–	≡	≡	+		
Ta = 90′	·0625	·0001	·05	0	0	0	0	0	0
	·25	·0001	·05	0	=	+			
Ta = 270′	·0625	·0001	·05	0	0	–	–	=	≡
	·25	·0001	·05	0	+				

TABLE IX. *Experiment VII.*

June 11, 1906.

	Toxin made up to ·5 c.c.	Antitoxin made up to ·5 c.c.	Brain in emulsion in 1 c.c.	Date and result 12	13	14	15	16	17
Ta = 0	·0125 c.c.	·00002 c.c.	·01 gm.	0	0	0	0		
	·025	·00002	·01	0	0	0	0		
	·05	·00002	·01	0	≡	≡	+		
	·1	·00002	·01	=	+				
Ta = 6 hrs.	·0125	·00002	·01	0	0	≡	≡	≡	+
	·025	·00002	·01	0	=	+			
	·05	·00002	·01	0	=	≡	+		
	·1	·00002	·01	–	≡	+			

In all cases the antitoxin had combined with so much toxin, that the fluid in each tube of the antitoxin series was much more toxic than the fluid in the corresponding control tube.

The results are given in Tables IV., V., VI., VII.

In the last experiment (Exp. V., Table VII.) the amount of toxin in the control series was not the same as that in the antitoxin series, but this amount less by the $L+$ dose.

The long time taken by toxin and antitoxin to enter into combination is well illustrated by this method. In Exp. VI. four series of tubes were prepared, in which the time allowed for the combination of toxin and antitoxin varied from zero to $4\frac{1}{2}$ hours. Table VIII. shows the result. It is only after a considerable time that our toxic compound has become stable enough to resist dissociation by the brain emulsion. Table IX. refers to a similar experiment.

Dissociation of brain-toxin compound.

Besredka (1903), in the paper already quoted, shows that the brain-toxin combination can be completely dissociated by means of antitoxin, and infers that antitoxin has a greater affinity for toxin than has the brain matter. Even so, both brain and antitoxin may owe their power of combining with toxin to the same side-chain. Organic chemistry is full of instances, where the affinities of a side-chain are altered in degree and not in kind by alterations in the radicle to which they are attached; instance the various chlor-acetic acids.

Besredka's experiment was as follows: To his "cerveau toxique" he added antitoxin and allowed the mixture to stand. The brain matter was then washed in the centrifuge, till the washings were free from antitoxin. It was then tested and found not only to be atoxic, but to have regained completely its protective power. The toxin had all gone over into combination with antitoxin. He used, however, an excess of antitoxin, though he does not state exactly how much.

Experiment VIII.

I repeated the experiment in a modified form, using a minimal quantity of antitoxin. The amount of antitoxin chosen was ·00032 c.c. the $L+$ dose for this quantity being ·1 c.c. of my toxin. ·1 c.c. of toxin was therefore mixed with ·2 gram of brain, in emulsion in 2 c.c. of saline, and the mixture was made up to 4 c.c. In a second tube ·2 c.c. of toxin and ·2 gram of brain were used. The mixtures were left for two hours at 10° C. The toxin remaining in solution in tube (1) would then be nearly nil, and in tube (2) considerably less than the amount required to kill a mouse in a dose of the volume generally used (*vide* Table V., control series).

To each tube was then added ·00032 c.c. of antitoxin, contained in 4 c.c. of saline, and the tubes were left for 8 hours at 10° C.

Now if the antitoxin has lost any of its protective power, it must have taken the toxin from combination of the latter with the brain matter; also there is enough toxin present to neutralise the whole of the antitoxin, so that, if the solution retains any protective power, we know that the brain matter has been able to hold in combination some of the toxin, in spite of the presence of unsaturated antitoxin.

The contents of the two tubes were centrifuged, and the supernatant fluids tested for antitoxin. It was found that unsaturated antitoxin was

present in each. Volumes of 1 c.c. were mixed with different quantities of toxin. Now we started with ·00032 c.c. antitoxin in each tube, and the volume in each tube was 8 c.c. Each cubic centimetre therefore corresponds to ·00004 c.c. of antitoxin, for which the L_0 and $L+$ doses of my toxin are respectively ·01 and ·0125 c.c. The tests are given in Table X. It will be seen that the fluid of tube (1) protected against ·005 c.c. of toxin, therefore that half the antitoxin was unsaturated with toxin, and that the brain matter had been able to retain in combination more than half the toxin which was given to it originally.

We cannot, therefore, argue that the combination of toxin with brain is of a different nature from the combination with antitoxin, on the ground that an excess of antitoxin can take all the toxin from the brain, to which it was previously bound. The toxin is divided between the brain and the antitoxin, and it is only in the presence of excess of the latter that increased mass-action enables it to take over all the toxin.

TABLE X. *Experiment VIII.*

October 4, 1906.

Fluid of tube (1)	Toxin (in 1 c.c.)	Date and result						
		5	6	7	8	9	10	11
1 c.c.	·005 c.c.	0	0	0	0	0	0	0
1	·0075	0	0	0	–	≡	≡	×
1	·01	0	0	=	≡	≡	×	
1	·0125	0	≡	≡	≡	+		
Fluid of tube (2)								
1	·005	0	0	–	=	=	=	≡
1	·0075	0	–	=	≡	≡	≡	+
1	·01	0	=	≡	+			
1	·0125	–	≡	+				

Moreover, there is already a large body of evidence showing that dissociation of a toxin-antitoxin combination can occur.

Behring and Ransom (1898) demonstrated this in the case of tetanus toxin and antitoxin, by dilution. Using a strong toxin, they prepared a mixture with antitoxin which was nearly neutral. The test mouse had very slight tetanus. This mixture was diluted 10 times, 100, 1000, and 10,000 times and the same volume of each dilution was injected into the test mice. The thousandth dilution caused death in three days, and all the dilutions were more toxic than the original mixture. If this mixture is allowed to stand for 24 or 48 hours before the dilutions are prepared, the effect is not obtained. The toxicity decreases gradually

as the dilution increases. After this time, therefore, the combination has become more stable.

R. Otto and Hans Sachs (1906) have lately confirmed these results.

Carl Bruck (1904), working with Wassermann, showed that if a toxin-antitoxin mixture be injected subcutaneously in the site of a previous injection of adrenalin, its toxicity is greater than in a control animal. He explains the result by stating that the antitoxin is forced to remain at the site of the inoculation, because of the vascular constriction, while the toxin which diffuses more readily, and travels along nerves, is removed from the sphere of influence of the antitoxin. He regards the experiments as a proof that dissociation occurs, and to a greater extent in the adrenalin animal than in the control; but it may be objected that the free toxin, in the absence of a normal circulation, is held at one spot in greater concentration, and is hence able to invade the axis-cylinders in larger quantities than when it is diffused through the body by the blood and lymph streams.

Finally J. A. Craw (1905 and 1906), following up Martin and Cherry's (1898) work with gelatine filters, succeeded in demonstrating the dissociation of a toxin-antitoxin compound in vitro. He used Myatherium lysin and antilysin, and passed neutral mixtures of the two through gelatine filters, under a pressure of 100 atmospheres. The antilysin has the larger molecules, or particles, and is held back by the filter while some of the toxin is forced through. A toxic filtrate is obtained.

The correspondences between brain and antitoxin in their reactions with tetanus toxin are seen to be fairly complete, but there remains the fact, that when we add brain emulsion to toxin, we do not obtain nearly so sharp a neutralisation point as we do on adding antitoxin. Exp. IX. (Table XI.) is an example of this. 1 c.c. of a 2 per cent. dilution of toxin was placed in each of 4 tubes. Different weights of brain, always emulsified in 1 c.c. of saline were added to each tube. Four

TABLE XI. *Experiment IX.*

October 22, 1906.

Toxin	Brain	Weight of guinea-pig	Date and result							
			23	24	25	26	27	28	29	30
·02 c.c.	·3 gm.	325	0	0	–	=	≡	≡	≡	≡
·02	·16	330	0	–	=	≡	≡	≡	≡	≡
·02	·08	325	0	=	≡	≡	≡	≡	≡	≡
·02	·04	350	0	=	≡	≡	≡	+		

guinea-pigs were used as test animals, and each had a dose of ·2 c.c. from a different tube. Though ·04 gram of brain was enough to delay death till the sixth day, ·3 gram was not enough to prevent altogether the onset of tetanus.

I am inclined to believe that this phenomenon is to be explained by the destruction of the brain particles within the tissues. Toxin might thus be set free, in the same way as ricin can be set free from its combination with fibrin by the aid of digestive ferments (Martin Jacoby, 1902).

Conclusions.

(1) The affinity of brain matter for tetanus toxin is specific, as is that of antitoxin.

(2) A solution of pure toxin is easily rendered innocuous by treatment with brain matter; but if a small dose of antitoxin has been added to the toxin some hours beforehand, treatment with brain matter no longer suffices to render the solution atoxic. The free toxin is removed, and we have isolated a toxic compound of toxin and antitoxin.

(3) Both brain-toxin combinations and antitoxin-toxin combinations dissociate with more or less rapidity, unless in the presence of enough free toxin (and free brain or antitoxin) to maintain the state of equilibrium. Consequently to obtain a neutral mixture one adds a large excess of brain or antitoxin beyond the combining equivalent. The dissociation is thus reduced till free toxin is no longer recognisable by our tests.

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Note on the occurrence of n. Octoic (Caprylic) acid in the butyric fermentation; and, the constitution of the hexoic acid in butter fat.

By H. S. Raper.

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(*a*) *The occurrence of n. Octoic (Caprylic) acid in the butyric fermentation.*

It has been suggested by Nencki, that in the butyric fermentation of lactic acid, the latter splits up into a molecule of formic acid and a molecule of acetaldehyde thus:—

$$CH_3 . CHOH . COOH = CH_3 . CHO + H . COOH.$$

Two molecules of aldehyde then unite to give butyric acid, C_3H_7COOH.

This suggestion has been developed by Magnus-Levy (*Arch. für. Anat. und. Physiol.* 1901), and by Leathes (*Problems in Animal Metabolism*, London, 1906, pp. 81—86), who suggest that by a further union of aldehyde molecules and ensuing reduction, higher fatty acids could be formed. The butyric fermentation would thus represent the first stage of a reaction which goes to completion when fats are formed.

The fact that n. Hexoic (Caproic) acid, $C_5H_{11}COOH$, is a regular product of the butyric fermentation, supports this view. None of the higher members of the series of naturally occurring fatty acids have been noted however, and it was to determine whether any of these were present in the products of the butyric fermentation that the present investigation was undertaken.

A kilo. of crude butyric acid residues was obtained from Kahlbaum and carefully fractionated. The greater part of the acid boiled below 215° and was found to consist of n. hexoic acid. 8 gms. were obtained boiling above 215° and of this 2·5 grams distilled at 225—232°. In order to determine whether this was caprylic acid, it was converted into the acid chloride by means of phosphorus trichloride, and this in turn into the amide by the action of ammonia. After crystallising from petroleum ether and then from hot water, the amide melted sharply at 105—106°, which coincides with the melting point of caprylamide.

On analysis:—0·1104 gm. gave NH_3 corresponding to 7·90 c.c. $\frac{N}{10}$ $NH_3 = 10·0$ % N. Calculated for $C_8H_{17}NO$. 9·8 %.

The fraction boiling at 215—225° was neutralised with baryta and the solution evaporated to 50 c.c. The barium salt which had separated was filtered off and the free acid obtained from it by means of hydrochloric acid. This was then converted into the amide which melted at 105–6° and was therefore caprylamide.

The solution containing the soluble barium salt was evaporated to dryness, dried at 120° and analysed. 0·3724 gm. gave 0·2356 gm. $BaSO_4 = 37·20$ % Ba. Calculated for barium caproate 37·77 % Ba.

The fraction 215—225° therefore consisted of caproic and caprylic acids. As this fraction would also contain any heptoic acid if present, one must conclude that it is not one of the products of fermentation.

The residue after the fractionation was too small to admit of further investigation.

(*b*) *The constitution of the hexoic acid in butter fat.*

The constitution usually assigned to the hexoic acid in butter fat is $(CH_3)_2CH . CH_2 . CH_2COOH$. That is to say, the acid is described as having a branched and not a straight chain of carbon atoms. It is thus different from practically all the other members of the series of naturally occurring fatty acids, which have a straight chain. Further, its formation could not be explained in accordance with the views of Leathes, as stated above. Chevreul (*Recherches sur les corps gras*, 1823), is generally referred to as having determined the constitution of the acid, but in the original work nothing could be found in reference to the constitution of the hexoic acid he isolated from butter fat.

In order definitely to settle the constitution of the acid, 400 gms. of butter were saponified with potassium hydroxide, and the acids liberated by means of sulphuric acid. The volatile acids were distilled off in a current of steam and were separated by fractional distillation.

The fractions boiling at 192—200°, and 200—210°, were converted into their acid chlorides and then in turn into the amides. After two crystallisations from water both melted sharply at 100°. The isocaproic acid usually stated to exist in butter fat boils at 197° and gives an amide melting at 120°; normal hexoamide (caproamide) melts at 100°. The hexoic acid in butter fat is therefore not anomalous in having a branched chain, but is normal hexoic (caproic) acid.

[From THE JOURNAL OF HYGIENE, Vol. VI. No. 5, October, 1906.]

BLOOD PARASITES OF THE MOLE,—INCLUDING A NEW FORM OF INTRA-CORPUSCULAR PARASITE.

(Plate X and One Diagram.)

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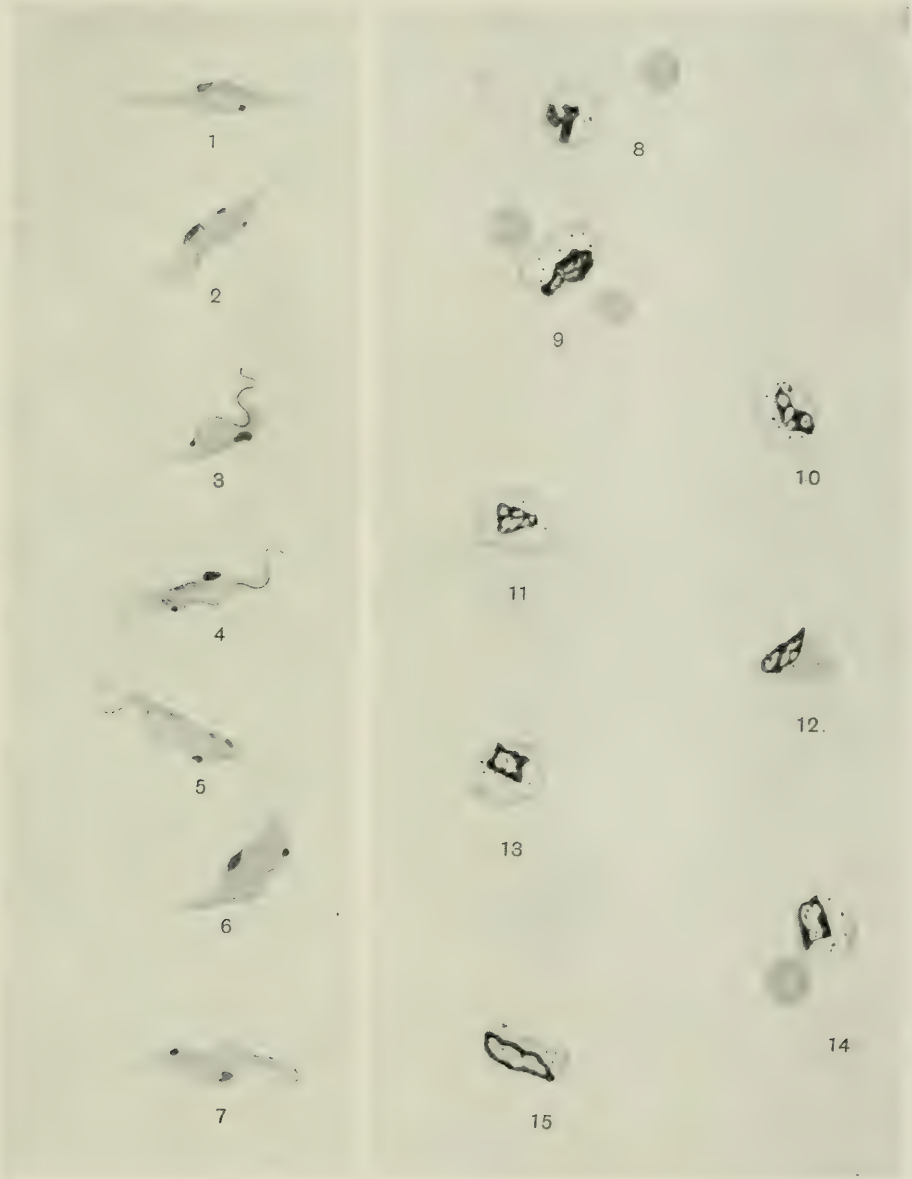
The material on which these notes are founded was obtained from moles captured in the neighbourhood of the Lister Institute at Elstree in Hertfordshire from the middle of May to the beginning of July, 1906[1]. In all 14 moles were examined.

1. *Trypanosomes.* Laveran and Mesnil (1904, p. 98, footnote) state that in 1845 trypanosomes, then described as vermicules, had been seen by Gros in the blood of moles and that they had not been seen since. Petrie (iv. 1905) observed a species of trypanosome in the mole which he believed to be new. This trypanosome he found in the blood of 6 out of 20 moles examined: but "the parasites," he says, "in every case were present in such small numbers that a satisfactory stained preparation has not yet been obtained." In the absence of stained preparations the morphological characters of the trypanosome found in the blood of the mole have, so far as I know, not hitherto been described. For the purpose of comparison with other trypanosomes the following average measurements are given and they are likewise represented in the accompanying diagram (p. 575).

From posterior extremity of body to centre of centrosome	9·3μ
From centre of centrosome to posterior border of nucleus	4·6μ
Nucleus	2·0μ
Anterior border of nucleus to anterior end of body	6·5μ
Free flagellum	5·2μ
Greatest depth of body	about 3·5μ

[1] Specimens were demonstrated before the Inaugural Meeting of the Pathological Society of Great Britain and Ireland in July, 1906.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Only adult forms have been observed and they are fairly uniform in size and shape. The most striking feature, perhaps, is the long, pointed, proboscis-like posterior extremity. Under certain circumstances it can apparently be partially retracted as shown in Plate X, Fig. 6. From its staining reaction it is evidently a prolongation of the body-protoplasm and not a posterior flagellum. The nucleus is small relative to the centrosome and lies close to the ventral border. Though it and the centrosome are usually sharply defined, both are occasionally obscured by groups of chromatin particles varying in size and irregular in shape as shown in Fig. 5. In Fig. 7 there is the appearance of a vacuole in front of the nucleus.

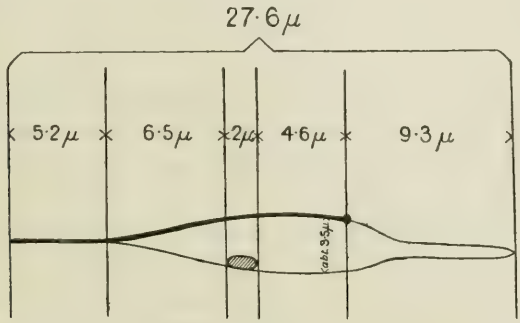

Diagram 1.

In the living condition this trypanosome is actively progressive, and though it has been seen to move short distances with its posterior extremity forward, it usually progresses with the flagellum to the front,—feeling its way, as it were, among the corpuscles.

Inoculation experiment. A white rat was inoculated intraperitoneally with blood containing living trypanosomes, but no infection followed.

Attempts at cultivation have so far proved unsuccessful.

2. *Bacillary-like bodies in the red blood corpuscles.* These are doubtless identical with the bodies described by G. S. Graham-Smith (x. 1905). His photographs (Plate XIV) leave no doubt as to the identity of the organism described by him and those here referred to. I have not yet seen anything comparable to the forms depicted in his coloured plate. I find that they stain with all the ordinary bacterial stains:—Carbol-thionin, dilute carbol-fuchsin, methylene blue, as well as by various modifications of the Romanowsky method. They are not acid fast and they do not stain by Gram.

Inoculations of the blood from the heart of an infected mole into a rabbit (intravenously), a rat and a guinea-pig (intraperitoneally), and a mouse (subcutaneously), all gave negative results.

The relative proportion of infected moles and the degree of infection seems to be much greater in the neighbourhood of Elstree than in that of Cottenham near Cambridge, where Graham-Smith found them. Out of a total of 102 moles examined by him 10 were found to be infected, and in only two of these ten was the infection large. Of 14 moles examined here during May and June of this year 12 were found to be infected, some largely so, and all to such a degree that there was no difficulty in finding infected corpuscles. No mention is made of the time of the year when Graham-Smith found his infected moles, so that it is just possible that the difference may be seasonal.

3. *New form of intracorpuscular parasite.* The form now to be described was found in two out of the 14 moles examined. As seen in stained preparations it is larger than a normal red blood corpuscle. The corpuscle in which it lies is stretched and reduced to a mere shell, and what remains of haemoglobin is altered, as indicated by its staining of a somewhat paler and more yellowish tint than adjacent normal corpuscles. The parasite itself varies in shape. When completely enclosed in the remains of a red blood-corpuscle it is more or less oval in outline. Where the corpuscle has become ruptured the parasite may assume various irregular amoeboid outlines, as seen in Plate X, Figs. 12 and 15. The body protoplasm stains by Giemsa's method a pale blue, while the nucleus gives the usual chromatin reaction. The nucleus varies in appearance. In some it is irregularly ring-shaped, the thickness of the ring too varying much in different parts. In others it is more diffuse, irregularly vacuolated or almost reticular in appearance. When ring-shaped or coarsely reticular, the parts within the ring or in the meshes of the reticulum usually stain pale blue like the body protoplasm. In the body protoplasm of most, but not of all, are to be seen small round sharply circumscribed particles almost black in colour (? Melanin).

Vassal (1905) describes an intracorpuscular parasite found by him in the blood of a squirrel (*Sciurus grisiemanus*), in Annam. Ring-forms, schizonts and gametocytes are described as being present in the blood at the same time; and the ring-forms are said to be indistinguishable from the ring-forms of the parasite of malignant malaria. The gametocytes of the parasite discovered by Vassal grow to about the same size as the parasite of the mole here described, though there are

morphological and other differences that need not here be detailed. Vassal attaches great importance to the resemblance between the parasite of the squirrel and that of human malaria, as suggesting the possibility of the squirrel being an alternative host to man for the parasite of malignant tertian fever.

In new countries where malaria attacks pioneers of civilization, and in those intensely malarious districts already occupied, where disturbance of the soil is followed by severe outbreaks of fever, it would be well in searching for the intermediary host in the absence of man, to examine the blood of animals such as the mole, that live and burrow in the soil.

In the blood of the two moles that harboured this parasite trypanosomes were also found, while they were the only two out of fourteen examined that did not show the bacillary-like form described by Graham-Smith. Trypanosomes were found associated with the bacillary-like forms in one case out of the twelve.

Smears from various organs.—Brain, bowel, bone-marrow, spleen, liver, lymphatic gland, etc., were examined but though prolonged search usually revealed a parasite or parasites in proportion to the number of blood cells present, no variation in the appearance was observed.

Though the material at disposal is insufficient to establish the life-history of this parasite, and though the protozoal nature of the bacillary-like forms first described by Graham-Smith may not be thoroughly established, certain points are of sufficient interest to raise the question of a possible relationship and to merit brief discussion. Granted that the number is small it is still a very significant fact that of a total of fourteen moles examined no less than twelve were found to be infected by the bacillary-like form, while the only two that were not so infected harboured the large amoeboid form. A great drawback to the further study of these forms will be the difficulty of keeping moles alive. They seem to die very readily in captivity. It is, therefore, interesting to note that the mole in which the large intracorpuscular parasite was first found escaped, but was recaptured (unfortunately killed) on the 15th day after its escape, and that in the blood films taken before and after, the same appearances were found. The parasite, therefore, must either have a life-cycle of 14 to 15 days or it remains in the form in which we have seen it for some considerable time.

Analogy with the crescent of the malignant malaria parasite might suggest that these large forms appear late in the infection, persist in

the blood after all former bodies have disappeared, subserve the interest of the species and require for their further development the intervention of an intermediary host. The fully developed crescent, however, is a resting form while the form we are now discussing,—if we may deduce this from appearances seen in stained preparations,—seems still capable of amoeboid movements. (Plate X, Figs. 12 and 15.)

It is hardly probable, though still possible, that the organism, whatever it is, had just completed its life-cycle in the period during which the mole was at large. On the other hand, it may be a parasite with a very long life-cycle, so that at the stage in which we have seen it no appreciable change is to be detected in 14 days. Allowing that the forms seen may be developing gametocytes they may still be stages in the life-history of some parasite of which no other stage has yet been seen. This large form must have entered the red blood corpuscle at a very much earlier stage and must have grown in its interior to the size we now see it.

The fact that 14 infected moles have been examined without our being able to demonstrate transition forms argues against the likelihood of a relationship between the bodies described by Graham-Smith and this new form. On the other hand, the evidence in favour of the view that this new form is a parasite *sui generis* rests so far on insufficient data. A much larger number of moles harbouring this parasite would require to be examined before reliable conclusions could be deduced. The association of this haemocytozoon with trypanosomes in the blood of the two moles in which it has been observed may be mere coincidence; but in view of Schaudinn's observations on *Trypanosoma noctuae* and of the relationship claimed by him to exist between endocorpuscular (*Halteridium*) forms and free (*Trypanosoma*) forms of that parasite in the blood of the owl (*Athene noctua*), further classification may well be delayed until something more is known regarding its life history.

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DESCRIPTION OF PLATE X.

TRYPANOSOMES OF THE MOLE.

Figs. 1, 3, 4, 6 and 7. Trypanosomes with centrosome and nucleus compact and sharply defined. Fig. 6 also shows the posterior extremity partially retracted; and Fig. 7 the appearance of a vacuole in front of the nucleus.

Figs. 2 and 5. Trypanosomes with diffuse chromatin-staining granulations in which the centrosome and nucleus are more or less obscured.

NEW FORM OF INTRACORPUSCULAR PARASITE FOUND IN THE MOLE.

Figs. 8—12. Parasites with irregularly-shaped nucleus showing various degrees of vacuolation; and with dark granules in the body protoplasm varying in number and arrangement. Fig. 12. Free parasite. In most cases the remains of the corpuscle is seen to surround the parasite.

Figs. 13—15. Parasites with nucleus irregularly ring-shaped. Fig. 15. Free parasite with remains of red blood corpuscle still clinging to one end.

NOTE.

Figs. 1, 3, 4, 6 and 7. From blood films fixed in methyl alcohol and stained by Giemsa's method.

Figs. 2 and 5 also 8—15. From blood films fixed in absolute alcohol and stained in a freshly prepared mixture of weak watery solutions of Giemsa's azur II and of eosin.

All specimens magnified 1200 diameters.

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UPON THE AGGLUTININ TEST IN THE DIAGNOSIS OF TUBERCULOSIS.

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In 1898 Arloing and Courmont[1] published the results of experiments made by them on the agglutination produced by the serum of tubercular patients. For this purpose they used a strain of the tubercle bacillus isolated by the former, which produced a uniform turbidity when grown in glycerine broth. With this culture, serum from tubercular patients gave reactions at dilutions of 1 in 5, 10 or 20 in a large percentage of cases, and that from normal individuals only in a much smaller percentage and then not very strongly. From this they concluded that the agglutination reaction was likely to be of considerable use in the diagnosis of tuberculosis.

Other observers, however, who tested these results both with Arloing's culture and with various other preparations of the bacilli, came to the conclusion that the reaction was of no practical use. Koch[2] for instance showed that with most uninoculated animals similar reactions could usually be obtained, but that when inoculated they often gave agglutinations up to 1 in 200, 500 or even 2000. On investigation of a series of hospital patients and others, tubercular and non-tubercular, he found the following:—

Non-tubercular. 1 case reacted at 1 in 50, 5 at 1 in 25, 6 at 1 in 10, 19 gave practically no result.

Tubercular. 1 case reacted at 1 in 50, 4 at 1 in 25, 14 at 1 in 10, 59 gave practically no result.

[1] *Comptes rendus de l'Acad. des Sciences*, 19 Sept. 1898, and *Gazette des Hôpitaux*, 1 Dec. 1900, p. 1467.

[2] *Deutsche Med. Wochenschr.* 28 Nov. 1901.

At the same time in cases which had been treated with tuberculin he often obtained reactions up to 1 in 300.

Considering the difference of opinion on the subject, it seemed of interest to test the power of agglutination during the progress of a case of tuberculosis from the beginning to the end, in order to find out whether there ensued any marked alteration in the reaction. Monkeys were selected for the experiment as being animals which very quickly acquire intestinal tuberculosis on feeding with the bacilli. Accordingly two monkeys, both apparently normal, were fed with an emulsion of a virulent culture of human tuberculosis, and the serum of each before feeding, and at various times afterwards, was tested against a preparation of the same strain of *B. tuberculosis* as that with which they had been fed. The preparation of tubercle bacilli used for agglutination experiments was made by grinding in a mortar a culture of the bacilli grown on glycerine potato, and making a thick emulsion of the rubbed up mass in water. By centrifugalisation it was possible to drive all but the smallest masses to the bottom, and still leave a considerable number of fairly well separated bacilli in the fluid. This fluid when pipetted off showed very little deposit and no sign of the formation of flocculi, even after four or five days. The examinations were made by the macroscopic method, one part of serum being mixed with ten or more parts of the emulsion, and the formation of flocculi down the sides or at the bottom of the tubes noticed after standing at 37° C. over night. Microscopic examinations were also made, but in no case could agglutination be observed with certainty at as high a dilution.

Monkey "A" was never as lively as the other, rapidly became thin, and died in about a month. The post-mortem examination showed an advanced condition of pulmonary phthisis, with cavities and tubercular pneumonia in the left lung. There was no ulceration of the intestine or any sign that the tuberculosis had been caused by the feeding. From this it seems likely that it was suffering from tuberculosis before the feeding took place.

Monkey "B" on the other hand lived rather more than two months, and being then in a dying condition was bled to death. On post-mortem examination it showed marked general tuberculosis, with ulceration of the intestine, masses of matted mesenteric glands, and miliary tuberculosis of the whole body, pointing distinctly to the fact that the disease had originated from the feeding.

Before feeding, the serum from both monkeys completely agglutinated and cleared the emulsion of bacilli at a dilution of 1 in 10, and

showed a reaction up to 1 in 40, that from B showing a slight reaction at 1 in 50, and this result remained fairly constant, about 1 in 40 or 50, during the period in which the animals were under observation.

Table showing the reaction of agglutination of the two monkeys A *and* B, *between the date of feeding and the time of death.*

	Monkey A						Monkey B						
Dilution	Before feeding	6th day	11th	17th	26th	30th	Before feeding	6th day	11th	17th	26th	35th	64th
1 in 10	+	+	+	+	+		+	+	+	+	–	–	+
20	+	+	+	+	+		+	+	+	+	+	+	+
25	+	+	+	+	+		+	+	+	+	+	+	+
40	+	+	+	+	?	died	+	+	?	+	?	+	+
50	–	–	–	+	–		?		–	+	–	+	?
60	–	–	–	–	–		–	–	–	–	–	–	–
100	–	–	–	–	–		–	–	–	–	–	–	–

The value of the emulsion of tubercle bacilli used was tested by the reaction produced on it by the serum of goats; one, which was normal, giving a slight reaction at a dilution of 1 in 20, and two, which had been inoculated with the juices of ground tubercle bacilli, giving marked reactions up to 1 in 80. Considering the fact that animals inoculated with these tubercle juices give such a marked reaction, it is interesting that those which have acquired tuberculosis in the usual manner by taking in the living organisms through the mouth, either into the lungs, or into the intestinal tract, show such small variation from the normal.

The above observations show that during the progress of a tuberculosis infection in these monkeys, no agglutination titrate of any diagnostic value could be obtained in the early or late stages of the disease.

My best thanks are due to Dr Allan Macfadyen, who kindly carried out the feeding experiments, for his constant advice and assistance.

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